American Journal of
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The Chemical Composition of Urinary Calculi from Range Steers

KARL F. SWINGLE, Ph.D.

Bozeman, Montana

OCCLUSION of the urethra by urinary calculi is responsible for the loss of a considerable number of range steers each winter in Montana and other western states. As a preliminary step in determining the etiology of these stones, a qualitative chemical analysis was made of calculi from 63 cases of urethral occlusion in Montana range steers.

There have been published relatively few chemical analyses of urinary calculi from cattle. Below are summarized the available publications of the last forty years which discuss the chemical composition of urinary calculi from cattle. Possibly in the older literature there are additional references.

In 1909, Law [1] discussed in a Bureau of Animal Industry report the problem of calculi in cattle. There is no bibliography in this report, but the author evidently had access to analytical data on urinary calculi in cattle. He described eight types of calculi, classified according to appearance and chemical composition. The first four types are largely calcium carbonate with varying amounts of magnesium carbonate, iron carbonate, and organic matter. The fifth type is primarily magnesium ammonium phosphate with calcium oxalate and organic matter and a little calcium and magnesium carbonate. Type 6 are siliceous calculi with carbonates of iron and magnesia and organic matter. Sometimes the carbonates predominate and the silica is a subsidiary component. The seventh type is composed of calcium oxalate with calcium and magnesium carbonates and organic matter. The last type is gravel and no chemical composition is mentioned.

Michael [2] analyzed the calculi from a Shorthorn bull and found them composed principally of calcium phosphate with larger or smaller amounts of all the urinary constituents as occlusions.

Larson and Baily,[3] analyzing the kidney stones from a cow fed alkali water, found the composition to be moisture, 2.8 per cent, organic matter, 15.8 per cent, silica, 76.9 per cent, calcium, 0.08 per cent, magnesium, 0.05 per cent, and iron, 0.006 per cent. They note that the alkali water contained almost no silica but that 49.4 per cent of the total mineral intake of the cow was silica. Of a total intake of 9.122 lb. of silica, 7.294 lb. were excreted with the feces and 0.016 lb. in the urine.

In India, Ranganathan [4] analyzed 23 individual stones from cattle for moisture, phosphate, calcium, magnesium, carbonate, and total nitrogen, and in addition some of them for oxalate and total ash. All of these stones were composed primarily of calcium carbonate with a small amount of magnesium carbonate. Except for two stones which had none, all had phosphate to the extent of less than 2 per cent P_2O_5. Nitrogen was detectable in all but one stone but was never over 1 per cent. His analyses in every case accounted for over 82 per cent of the dry weight of the stone. He did not analyze for silica, but it is evident from his data that there could not have been much silica in any of these calculi. Uric acid was not present in any stones. These animals were fed various types of Indian feeds, but only one had been grazing. In a later report, Ranganathan [5] described a different type of bovine calculus. This stone was 16.7 per cent moisture, 7.6 per cent nitrogen, 22.0 per cent P_2O_5, 10.9 per cent CaO, 12.5 per cent MgO, and 0.00 per cent CO_2. There was no uric acid. He says that the nitrogen was present neither in the form of uric acid nor as an ammonium compound.

Blount [6] lists seven types of calculi in cattle, namely, silicates, calcium oxalate, xanthine, calcium and magnesium carbonates, calcium and magnesium phosphates, triple phosphates, and iron carbonate.

Pribyl [7] made a study of the silica content of blood serum of several species including cattle. He did not analyze any stones, but he stated that part of the reason for the study was in relation to the problem of calculus formation, thereby implying that he was familiar with silica stones.

Contribution from the Montana Veterinary Research Laboratory, (Montana Experiment Station and Livestock Sanitary Board coöperating) Agricultural Experiment Station, Montana State College, Bozeman. Paper No. 286 journal series.

The author wishes to express his appreciation to Mrs. Frances Strickfaden and to Mrs. Barbara Noel for technical assistance in conducting many of the analyses reported in this paper.

Woodruff[9] in discussing the incidence of urinary calculi in feedlot cattle, states that urinary calculi are usually composed of uric acid, calcium oxalates, or calcium phosphates. He does not give the source of his information. He is the only author who includes uric acid in the composition of bovine urinary calculi.

Aston,[7] in reporting calculi from 2 animals in New Zealand, described the first as being chiefly $MgNH_4PO_4$, fat, and pigment. The second was SiO_2, $CaCO_3$, cystine, and calcium phosphate. This is the only reference to either fat or cystine in urinary calculi of cattle.

Newsom[10] states, "Practically all analysts agree that the calculi of cattle and sheep are composed of calcium, magnesium, and aluminum salts of phosphoric acid. The studies by our own chemistry department confirm these views." Newsom was discussing feedlot cattle.

Mathams and Sutherland[11] found that kidney stones taken from 3 slaughter cattle at Brisbane, Australia, were almost pure silica. A fourth stone was also largely silica but contained a trace of calcium oxalate. They suggest that this stone may have consisted of a nucleus of calcium oxalate upon which was deposited the silica. All four stones were from aged dairy cows on pastures containing a great deal of blady grass, *Imperata cylindrica*, which has a high silica content.

A report from India by Sinha[12] describes three calculi which were analyzed and found, like those of Ranganathan (quoted above), to consist of calcium and magnesium carbonate, with traces of phosphate.

None of these reports refer to cattle raised under the same conditions as those which are included in this study, namely steers wintered on the ranges of western America with little or no supplementary feed other than hay.

SELECTION OF SAMPLES

The calculi analyzed in this study have been collected in Montana over the past six years. Some were taken at operation for the relief of "waterbelly" cases but most of them at autopsy of such cases. Included in this report are only those stones which came from cases of occluding calculi among range steers. Strictly excluded are any stones from feedlot animals, from female cattle, from bulls, from animals known to be suffering from other disease, and from slaughter animals not suffering from urethral occlusions. In several cases, in addition to the calculi causing urethral obstruction, calculi from other parts of the urinary tract were collected and analyzed, and the results of these analyses are included in a special part of this report.

The stones were mostly light gray to white and of the "mulberry" type, hard, brittle, and lamellar. A few typical stones are shown in figure 1.

All of the stones were ground very finely in a mullite mortar before analysis.

ANALYTICAL PROCEDURES

The procedures used in these analyses were, with the few exceptions noted, developed and tested in our own laboratory. They involve no new ideas and represent the simplest procedures compatible with reliability of the results. They are described in detail here for whatever value they may be to others making similar studies. The choice of ions to be sought is based purely on what we considered to be the most probable components of these stones. It is quite possible that some components may have been overlooked. Indeed, this is probable since in some cases the analytical results fail to show a cation-anion balance (unless it is considered that the protein can function as either cation or anion).

Fusion.—The detection of Ca, Mg, and Si (and also observation of charring) was preceded by a sodium carbonate fusion. For this purpose, approximately 5 mg. of the powdered stone was mixed with about 20 mg. of sodium carbonate in a small platinum crucible and fused completely. (During the early part of the heating, observation of blackening and giving off a burnt odor was evidence of the presence of organic matter.) The fused cake was taken up in a minimum of dilute acetic acid (8 N). A drop of this solution was used for the calcium detection, a drop for the magnesium test, and the remainder for the silica detection.

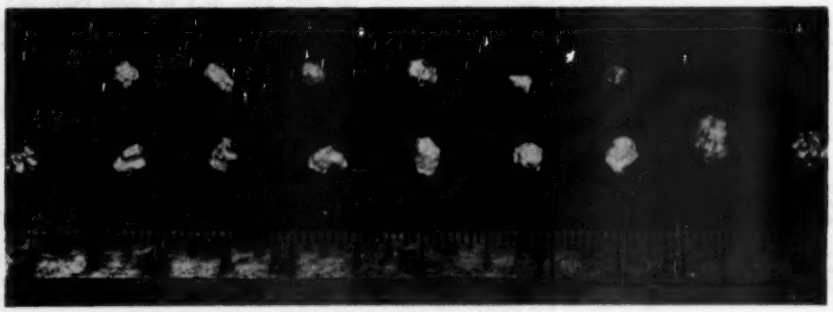

Fig. 1—Typical occluding urethral calculi from range steers.

Calcium.—A drop of the fusion solution was placed on a microscope slide. A drop of dilute sulfuric acid (3 N) was placed near it. With a platinum wire the sulfuric acid drop was drawn into the unknown drop and the formation of calcium sulfate crystals observed under the microscope. These crystals are long, slender needles, singly, or gathered into sheaves, x's, or stars. They may later broaden into masses of thin plates.

This is the least reliable of the methods we used, and when the amount of calcium is very small it may be missed.

Magnesium.—A drop of the fusion solution was placed on a spot plate. A drop of S & O reagent * was added and then a drop of 10 N sodium hydroxide. On mixing, the formation of a flocculant precipitate colored blue by the dye was an indication of magnesium ion.

Silicon, (Adapted from Bunting [33]*).*—The remainder of the fusion solution was transferred to a small test tube, and a drop of acid molybdate solution ** was added. After five minutes, 4 drops of 10 per cent tartaric acid solution was added. One drop of ANSA solution † was added. The development of a blue color within ten minutes indicated silicate.

Ammonium Ion.—A portion of the powdered stone was mixed with some sodium carbonate on a spot plate. A hanging drop of Nessler's reagent was prepared on a cover glass. A drop of water was added to the mixture on the spot plate and the cover glass with the hanging drop of Nessler's reagent immediately placed over it. A yellow to red color or precipitate developing in the Nessler's reagent indicated ammonium ion in the sample.

Protein.—A drop of 10 N sodium hydroxide was placed in the depression of a spot plate. A little of the powdered stone was dropped into this drop, and immediately placed over it was a cover glass with a hanging drop of Nessler's reagent. The spot plate was placed on a hot plate for a few minutes to observe any coloration of the hanging drop due to the release of ammonia from protein in the sample. (Ammonium ion interferes, but in our series of samples ammonium ion was so rarely encountered that this interference was of no consequence; urate ion does not interfere.)

Phosphate.—Two or 3 drops of 1 N nitric acid was added to a bit of the powdered stone on the spot plate. A drop of ammonium molybdate solution (same as used for the silicon test) was added. A yellow color was indicative of phosphate.

Oxalate, (Adapted from Dorronsoro and Fernandez [34]*).*—A bit of the powdered stone was mixed with a little resorcinol on a spot plate. A drop of concentrated sulfuric acid was added and allowed to stand several minutes. A greenish blue color indicated oxalates.

TABLE 1 — The Qualitative Composition of Urinary Calculi from Range Steers

Animal No.	Protein	Si	Ca	C_2O_4	Mg	NH_4	CO_3	PO_4
2605-1	+	+	+	+	+	—	+	—
2605-2	+	+	—	—	—	—	—	—
2605-3	+	+	—	—	—	—	—	—
2605-4	+	+	—	—	—	—	—	—
2605-5	+	+	+	—	—	—	—	—
2605-6	+	+	—	—	—	—	—	—
2605-7	+	+	—	—	—	—	—	—
2612	+	+	+	t	—	—	—	—
2693	+	+	+	—	—	—	—	—
2695	+	+	+	+	+	—	—	—
2696A	+	+	—	—	+	—	—	—
2703	+	+	+	—	—	—	—	—
2711	+	+	—	—	—	—	—	—
2720	+	+	+	+	—	—	—	—
2721	+	+	+	+	—	—	—	—
2725	+	+	—	—	—	—	—	—
2726	+	+	+	+	t	—	—	—
2728	+	+	—	—	—	—	—	—
2730	+	+	+	—	—	—	—	—
2738	+	+	+	t	+	—	—	—
2729	+	+	+	—	—	—	—	—
2741	t	—	—	—	+	+	—	+
2742	+	+	+	+	—	—	—	—
2746	+	+	+	t	—	—	—	—
2747-1	+	+	—	—	—	—	—	—
2747-2	+	+	+	—	—	—	—	—
2748	+	+	+	—	—	—	—	—
2749	+	+	—	—	—	—	—	—
2754-1	+	+	+	+	—	—	—	—
2759	+	+	+	+	—	—	—	—
2760	+	+	+	+	+	—	—	—
2761-2	+	+	+	+	—	—	—	—
2766	+	+	+	t	+	—	—	t
2767	+	+	—	—	—	—	—	—
2774	+	+	—	—	—	—	—	—
2780-2	+	+	+	+	+	—	+	—
2887	+	+	+	—	—	—	—	—
2893	+	+	—	—	—	—	—	—
2902	+	+	—	—	—	—	—	—
2904	+	+	+	+	—	—	—	—
2924	+	+	—	—	—	—	—	—
2925	+	+	+	—	—	—	—	—
3149-2	+	+	—	—	+	—	—	—
3180	+	+	—	—	—	—	—	—
3185	+	+	—	—	—	—	—	—
3187-2	+	+	—	—	+	—	—	—
3195-4	+	+	—	—	—	—	—	—
3196-1	+	+	—	+	—	—	—	—
3220	+	+	+	+	—	—	—	—
3279	+	+	+	+	+	—	+	—
3656-2	+	+	—	—	—	—	—	—
3696	+	+	+	—	t	—	—	t
3670	+	+	+	+	—	—	—	—
3723	+	+	+	+	+	—	—	—
3771	+	+	+	+	—	—	—	—
4010-1	+	+	t	t	—	+	—	—
4030-3	+	+	—	+	—	—	—	—
4093	+	+	—	+	t	—	—	—
4134-1	+	+	—	—	—	+	—	—
4134-2	+	+	+	+	—	+	—	—
4201	+	+	+	—	t	—	—	—
4205	+	+	—	—	—	—	—	—
4259	+	+	+	+	—	—	—	—

+ — component present; t — component present in barely detectable traces; — — component absent.

Aluminum and urate were also tested for, but both were absent in every case.

* Dissolve 1.2 Gm. of o-p-dihydroxymonaso-p-nitrobenzene in 250 ml. of 0.25 N. sodium hydroxide.

** Dissolve 7.5 Gm. of ammonium molybdate in 50 ml. silica-free water and acidify with 32 ml. of 10 N sulfuric acid, and finally dilute to 100 ml.

† (A) Dissolve 9.0 Gm. sodium bisulfite in 80 ml. of silica-free water. (B) Dissolve 0.7 Gm. anhydrous sodium sulfite and 0.15 Gm. 1-amino-2-naphthol-4-sulfonic acid in 10 ml. silica-free water. Mix solution A and solution B and dilute to 100 ml. with water.

Carbonate.—A bit of the powdered stone was placed on a microscope slide. A cover glass was placed over it and the microscope focused on the powder. A drop of dilute acetic acid was allowed to flow under the cover glass. The development of growing bubbles indicated carbonate.

TABLE 2—The Qualitative Analysis of Urinary Calculi from Various Organs of Range Steers

No.	Organ	Protein	Si	Ca	C_2O_4	Mg	NH_4	CO_3	PO_4
2696A	Urethra	+	+	—	—	+	—	—	—
2696B	Bladder	+	+	+	—	—	—	—	—
2753-1	Kidney (not analyzed)								
2753-2	Ureter	+	+	—	—	t	—	—	+
2754-2	Kidney	+	+	—	—	—	—	—	—
2754-1	Urethra	+	+	+	+	—	—	—	—
2761-1	Kidney	+	+	+	t	—	—	—	—
2761-2	Urethra	+	+	+	+	—	—	—	—
2780-3	Kidney	+	+	+	+	+	—	—	—
2780-1	Bladder	+	+	+	+	+	—	—	—
2780-2	Urethra	+	+	+	+	+	—	+	—
3149-1	Kidney	+	+	—	—	+	—	—	—
3149-2	Urethra	+	+	—	—	+	—	—	—
3159	Bladder	+	+	—	—	—	—	—	—
3187-1	Bladder	+	+	—	—	+	—	—	—
3187-2	Urethra	+	+	—	—	—	—	—	—
3195-2	Kidney	?	+	+	—	—	—	—	—
3195-3	Bladder	+	+	—	—	—	—	—	—
3195-1	Urethra	+	+	+	—	—	—	—	—
3195-4	Urethra	+	+	—	—	—	—	—	—
3196-4	Kidney	+	+	—	+	+	+	—	—
3196-3	Bladder	+	+	—	—	—	—	—	—
3196-1	Urethra	+	+	—	+	—	—	—	—
3196-2	Urethra	+	+	+	—	+	—	—	—

+ — component present; t — component present in barely detectible traces; — — component absent.
Aluminum and urate were absent from all stones.

Urate.—To a bit of the powdered stone on the spot plate was added a drop of concentrated nitric acid and warmed to dryness on the hot plate. A red color indicated urate.

Aluminum.—A bit of the powdered stone was placed in a small platinum crucible. Two drops of concentrated sulfuric acid were added and then gently fumed off over a period of two or three minutes. The crucible was heated to redness for about thirty seconds and cooled. Two drops of 1:1 acetic acid and 1 drop of 0.1 per cent aluminon (aurin tricarboxylic acid) were added and the mixture warmed for a minute or two on a hot plate but not evaporated to dryness. Five drops of 3.2 N ammonium carbonate were added. Any red-colored precipitate indicated aluminum. This method would detect 0.5 μg. of aluminum even in such refractory materials as aluminum oxide abrasives.

RESULTS

The results of these 63 analyses are tabulated, stone by stone, in tables 1 and 2.

From table 1 it is seen that every stone contained protein; all but one also contained silicon. The one exception was a magnesium ammonium phosphate stone, the only one in this series.

Calcium oxalate was commonly present also, being found in 24 stones, but there were also 11 stones containing calcium but no oxalate and three stones containing oxalate but no calcium.

Magnesium, when found, was generally associated with calcium oxalate (9 stones), but in 3 cases silicate was the only associated mineral. There was one typical magnesium ammonium phosphate stone and two others in which traces of phosphate were detected with the magnesium (but ammonium was not detected). One stone showed magnesium and oxalate and another magnesium and calcium without any anion other than silicate.

The three occurrences of carbonate were all with both calcium and magnesium.

Ammonium ion occurred twice with calcium oxalate and once with only silicate. Aluminum was not detected in any stone. Urate stones did not occur in this series.

Table 2 records the results of analyses of stones taken simultaneously from different organs of the same animal. It is interesting to note the consistent appearance of protein and silicon in all these stones. At the same time, there are several cases where the components other than protein and silicon show some variation among the stones from different organs of the same animal.

In table 3 are recorded some incomplete quantitative analyses of a few calculi from range steers. Seven of these stones also appear in table 2. With the exception of No. 2605-1, these calculi all contained more than 50 per cent silica.

DISCUSSION

Even though there may be some components missed in a few of these analyses,

TABLE 3—Partial Quantitative Analyses of Urinary Calculi from Range Steers

No.	Moisture (%)	Ash (%)	SiO_2 (%)	CaO (%)	MgO (%)	P_2O_5 (%)
2574		70	> 50	present		
2605-1			11.7			
2605-2			50.6			
2605-3			69.6			
2605-4			73.3			
2605-5			51.8			
2605-6			75.8			
2605-8	2.02	76.92	69.85	0.55	0.18	0.85
2606	2.0	75.3	69.9	0.4	0.1	0.4
2612			64.1			

due to the very small size of some of the samples, in general the tables give a correct idea of the distribution of the ten components for which these calculi were examined.

The most striking observation, of course, is the universal appearance of protein and of silicon in these stones. Twenty of the 63 stones appeared to contain nothing but protein and silica, and some of the others probably contained only traces of other components. This raises the question of the possibility of the formation of insoluble silicic acid-protein complexes. A Russian reference of 1940 [13] described insoluble protein-silica complexes formed when certain wheat flour protein fractions are allowed to react with silica solutions. That similar complexes may occur in urine is quite possible since bovine urine normally contains 50 to 150 parts per million of soluble silica and also considerable quantities of mucin. We have not usually found albumin in the urine of calculus cases and never in normal steer urines, so the protein of these stones is probably mucoprotein, though the possibility of the temporary appearance of other proteins such as albumin or cellular debris has not been ruled out, but we have never observed it. It may be possible by analytical means to determine something of the nature of the protein in these calculi, but this has not yet been done.

With regard to the presence of silica in these stones, the following observations are made: Silica is a normal constituent of plants and a portion of this vegetable silica is absorbed by the ruminant and excreted in the urine. Analysis by us of a considerable number of steer urine samples has shown the presence of 50 to 150 parts per million of dissolved silica. The solubility properties of silica and the silicates are extremely complex and very little understood. Silica and the silicates form colloidal dispersions in aqueous mediums, and perhaps also true solutions. The conditions which bring about coagulation, precipitation, and crystallization of these dispersions and solutions are not well understood. Probably decreasing the amount of water, increasing the electrolyte concentration of the solution, and appropriate changes in pH all will contribute to the precipitation of silica. In addition, the presence of particulate "nuclei" may initiate the accumulation of silica. Strong alkalis tend to form soluble silicates with silica, and strong acids will precipitate silica as silicic acid, but within the pH range of urine these effects would not be encountered.

At present, it seems most probable that the stones are the result of a precipitation of the silica by the mucoprotein of the urine. We do not know the conditions determining whether the precipitate is dispersed or gathered into concretions, but we suspect at least two factors are the presence of particulate nuclei and alterations in the quality and quantity of protective colloids in the urine.

With regard to the components of these stones, other than protein and silicon, the very fact that there is some variation from one case to the next indicates that the ones found in any particular stone are those which happened to be present in precipitable concentrations at the time the stones were forming. Since calcium is always present in the urine, oxalate stones would be formed at times when the animal is excreting oxalate.

In the cases where calculi were available from several organs of the same animal it appears significant that, aside from the usual presence of protein and silica, the remaining ions were frequently not the same in the stones of the different organs of the same animal. We suggest that this may be due to the fact that the stones were formed at different times and that the ions most precipitable at the time were the ones that were occluded as the stone developed.

The absence of phosphate stones is not surprising, since bovine urine contains phosphate only at the time of very high levels of phosphate intake, and range cattle are usually not receiving much more than their minimum phosphate requirements.

Magnesium ammonium phosphate, as demonstrated in only one of these stones, is probably the result of a urinary infection which causes the kidneys to excrete phosphate and also splits urea to provide the ammonia. Probably this stone had an entirely different etiology from the other 62.

These analyses do not contribute much directly to the solution of the practical problem of water-belly among range steers. The seasonal nature of the malady, occurring as it does in mid-winter, suggests a nutritional factor, and both vitamin A and water deficiency have been implicated. The former may provide bits of desquamated epithelium as nuclei for stone formation, and the latter may result in increased con-

centration of the insolubles which make up the stones. An attempt was made in this laboratory [15] to produce urinary calculi in a few steers by severely limiting water consumption, but no calculi were formed, even in the animals receiving large amounts of soluble silicates in their ration. The low incidence of the disease, even in affected herds, is likely due to the passage of the calculi before they are large enough to cause occlusion of the urethra. The absence of clinical cases or urethral calculi in heifers and cows is probably not due to any difference in urinary chemistry, but to anatomical differences in the urethra, permitting much larger objects to pass without lodging in the passage. It is believed by practitioners that occluding calculi are less frequent in bulls than in steers. If this is the case, the explanation may lie in differences in the dimensions of the urethra or in the greater force of urination in the bull.

SUMMARY

Qualitative analyses of the stones from 63 cases of occluding urinary calculi in range steers revealed the universal occurrence of protein and silica. Frequently associated with these two components were calcium and oxalate and occasionally magnesium. Only rarely were ammonium, carbonate, and phosphate ions found. Aluminum and urate were not detected.

The qualitative analytical methods used for these components are described in detail.

The significance of these observations to the etiology of calculus formation is discussed.

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Multiple Schwannomas of Cattle
(Nerve Sheath Tumors; Multiple Neurilemmomas; Neurofibromatosis)

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NEOPLASMS of the peripheral nervous system derived from cells of the neural crest in domesticated animals have been recognized but not extensively studied in the United States.

In man, many detailed investigations have been made of tumors of this group, which are established as being related to Schwann's cells.[4, 8, 9, 12] Interest in von Recklinghausen's disease (multiple neurofibromatosis) and, in recent years, in tissue culture studies[9, 10] has stimulated much of this work. European investigators[7, 12] have described a limited number of well-documented cases of tumors of the peripheral nervous system in cattle whose cellular structure resembles a group of tumors in man believed to originate from Schwann's cells. Similar neoplasms have been reported in cattle in South Africa.[6]

It would seem appropriate that a description of a type of Schwann's cell tumor in cattle, and its potentiality to appear in a widely disseminated form, would help clarify the histological classification of soft tissue growths.

Although a minority of writers believe that all Schwann's cell tumors are basically fibrous mesodermal neoplasms, the generally accepted concept today supports the theory that they originate from the neural crest.[4, 8, 9, 12] The ability of Schwann's cells to form connective tissue fibers in tissue cultures suggests that, in addition to the neurilemma of all peripheral nerves, the endoneurium and perhaps even the perineurium may originate from these cells. This characteristic probably accounts, in part, for the connective tissue component of the closely related or partially synonymous neuromas, neurofibromas, and neurilemmomas. For clarity in separating schwannomas from fibromas and fibrosarcomas, it is necessary to accept as schwannomas only those tumors which still retain recognizable Schwann's cell structures. The same, but often difficult, evaluation of relation to Schwann's cells must be made of malignant tumors of this group which are designated "malignant schwannomas." The term "neurogenic sarcoma" is not used in keeping with current literature as it implies an origin from the mesoderm, and there is no evidence that mesodermal elements of the nerve sheath produce malignant fibrous tumors and certainly not nerve, as might be indicated etymologically.

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LITERATURE CITED

Joest[7] in 1924 summarized much of the early European work on this group of tumors in cattle and preferred the term "false neuroma." (False neuroma was the term originally used by Virchow in distinguishing tumors involving portions of nerves but not arising directly from nerve cells.) Joest cites Bossert as observing tumors in the following anatomic locations: axilla, 16; nerves of tongue, 2; nerves of larynx, 1; phrenic nerve, 1; musculocutaneous nerve, 1; sternal nerve, 1; intercostal nerve, 5; abdominal portion of the sympathetic nerve, 3; and 5 each in the cervical portion of the vagus and sympathetic nerve. Bossert found the median and radial nerves involved four times and the ulnar six times among 16 animals in the axillary group. Joest also mentioned Schlegel's group of 19 cases; the brachial plexus was involved in 15 of these (bilateral in 11 and unilateral in 4 cases). Schlegel observed the tumor once each in the nerves of the cervical, thoracic, and abdominal sympathetics. In 8 of the cases where the brachial plexuses were affected, the neoplasms occurred in other nerves. In later studies, Schlegel listed the order of frequency of involvement of nerves as: (1) brachial region (usually bilateral), (2) intercostal nerves, (3) sympathetic nervous system (cardiac, coronary, aortic, hepatic, splenic, and mesenteric plexuses), (4) skin and muscle of the leg, (5) head, (6) neck, (7) thorax, (8) shoulder, (9) back, and (10) ischiatic nerve. One animal that exhibited severe clinical manifestations had involvement of the cervical sympathetics, the aortic and cardiac plexsus, and the intercostal nerves. Joest observed the tumor in calves and supported the view that the neoplasm was a true hamartoma.

In 1936, Jackson,[6] in South Africa, reported 9 cases of false neuromata in cattle. The locations of these tumors were brachial plexus, 3 (in 1 the heart was also affected); intercostal nerves, 1; sciatic nerve, 1; tongue, 2; and 2 cases in which the site was not stated.

Peyron and Drieux[12] in 1937 compared 7 cases of neurofibromatosis observed by them in cattle with neurofibromatosis of man. They noted that skin lesions were not found in their cases in cattle although all had multiple lesions; most of these cattle had involvement of one or more of the visceral plexuses and particularly the plexuses of the heart.

Hutyra, Marek, and Manninger[5] described the clinical appearance of a limited number of animals afflicted with this disease.

OBSERVATIONS ON CASES STUDIED

The Pathological Division of the U. S. Bureau of Animal Industry, in its studies of neoplastic diseases of food-producing animals at the Denver Branch Pathological Laboratory, has collected 25 examples of a type of tumor of peripheral nerves in cattle which we prefer to designate as multiple schwannoma. We could not establish clear-cut criteria in this group of neoplasms to justifiably further classify the cases as neurofibromas or neurilemmomas. (Multiple lesions would vary individually so that diagnoses of both neurofibroma and neurilemmoma might often be made in a single case. Such diagnoses would be confusing if we are to consider them manifestations of a single neoplastic disease.) The term "neurilemmoma" was derived from the Greek word for nerve sheath and introduced by Stout [13] to indicate encapsulated tumors of nerve sheaths composed histologically of Antoni type A and B tissue. Stout's concept [11, 14, 15] of a benign, encapsulated neurilemmoma and a more potentially malignant neurofibroma seems well established in man and is valuable in the consideration of individual surgical specimens as a guide for their prognosis. However, Ackerman [1] probably touches on a problem in man similar to what is presented later in this paper when he states that the difference between neurofibromas and neurilemmomas are more clear-cut in tumors of the peripheral nerves than in neurogenous tumors within the thorax, and exact classification may be difficult. Foot [2] also emphasized the difficulties of classification of the human neurofibromas. Although he observed the peripheral tumors of von Recklinghausen's disease to be consistently similar in general structure, the frequency with which other tumors were associated with neurofibromatosis in the visceral tissues was disturbing to him. Concurrent neurilemmomas, leiomyomas, or other sarcomas were found so often that he postulated a relationship of these tumors to neurofibromatosis. He quotes the suggestions of other investigators regarding the derivation of the tumor from some inherent fault in the architecture of the covering membranes of the nervous system during embryonal life.

Most of the tumors reported in our series were found in slaughtered animals at abat-

TABLE 1—Anatomic Locations of Tumors Believed Derived from Schwann's Cells and Found Associated with or Incorporated in Groups of Nerves or Visceral Organs

Case No.	Estimated age (yr.) and sex		Brachial plexus	Intercostal nerves	Heart	Intrathoracic nerves, ganglia, and plexuses (except heart)	Cervical nerves, ganglia, plexuses	Intra-abdominal nerves, ganglia, plexuses	Intrathoracic and intra-abdominal lymph nodes	Nerves of the voluntary musculature
14871	7	F	x	.....	x	.....	.....	x	.....	x
17518	6	F	x	.....	.....	.....	.....	x	.....	.....
14675	10	F	x	x	.....	x	.....	x	.....	.....
14778	2	S*	.....	.....	x	.....	.....	.....	.....	.....
15015	2	F	.....	.....	x	.....	.....	x	.....	.....
15078	6	F	.....	.....	x	x	.....	.....	.....	.....
15512	6	F	.....	x	.....	.....	.....	.....	.....	.....
17363	5	F	.....	.....	x	x	.....	.....	.....	.....
17418	4	F	x	x	x	x	.....	.....	.....	x
17463	4	F	x	.....	x	x	.....	.....	.....	.....
17269	2	S*	x	.....	.....	.....	x	x	.....	x
D 121	6	F	.....	.....	x	.....	.....	.....	.....	.....
D 155	6	F	.....	.....	x	.....	x	x	.....	.....
D 345	9	F	.....	.....	x	.....	.....	.....	.....	.....
D 688	6	F	.....	x	.....	.....	.....	.....	.....	.....
D 874	9	F	x	.....	x	.....	.....	.....	.....	x
D 918	5	F	x	x	.....	.....	.....	.....	.....	.....
D 1208	5	F	.....	.....	x	.....	.....	.....	.....	.....
D 1341	6	F	.....	.....	x	x	.....	.....	.....	.....
D 1394	8	F	x	x	x	x	.....	.....	.....	.....
D 1527	aged	F	.....	.....	x	.....	.....	.....	.....	.....
D 1647	aged	F	.....	x	.....	x	.....	.....	.....	.....
D 1794	10	F	.....	x	x	x	.....	.....	x	.....
D 1829	6	F	x	.....	.....	x	x	.....	.....	.....
D 2231	7	F	.....	.....	x	.....	.....	.....	.....	.....
Totals			10	8	17	10	3	6	1	4

* Steer; F—female; x—microscopic verification of a suspected Schwann's cell tumor.

toirs under federal inspection and were submitted by the Meat Inspection Service for histological examination. Records of other cases of multiple schwannomas where tissue sections were no longer available for review were not included in this report. Although several fibromas and fibrosarcomas of the skin were studied in our collection of bovine tumors and some showed a tendency toward whorling or palisading, none had a clearly defined Schwann's cell structure which would identify them as schwannomas. The lack of skin lesions in our material would suggest that neurofibromatosis of man, while similar in many of its manifestations to the multiple Schwann's cell tumor of cattle, is a disease peculiar to man and dependent, perhaps, on genetic or developmental factors. Two cases of the closely related acoustic tumors not included in this series will be reported in a subsequent paper. These benign, solitary tumors of the acoustic nerve, while possessing a Schwann's cell element and probably having a similar derivation, deserve separate consideration because of their occurrence to date as solitary tumors and the interest in a similar tumor in man.

Table 1 lists the occurrence of the tumor in various groups of peripheral nerves. The brachial plexus, heart, and intercostal nerves were the most common anatomic sites of the growths, and lesions were found in one or more of these locations in the 25 cases. Case D 1394 (fig. 1, 2, 3) is 1 of 2 cases in which there was involvement of all three sites. The lesions in the brachial plexuses were frequently bilateral and easily recognized by the extensive involvement of one or more nerves. A typical plexiform schwannoma of the brachial plexus is shown in figure 3. Tortuosity and nodular proliferation are evident in these involved nerves. The nodular or knotty thickening of the nerve is often shiny or glistening. Other tumors, usually encapsulated when attached to or replacing a portion of a nerve, were greyish white, firm neoplasms with pinpoint areas of softening and liquefaction. Many of the tumor masses in the heart, mediastinum, and abdomen were soft, greyish tumors and were thinly encapsulated or nonencapsulated. On section, they were often lobulated and frequently a lobule or an entire lesion would have a slightly gelatinous consistency. Areas of hemorrhage, necrosis, and mucoid change occasionally gave the tumors a mottled appearance.

The mediastinal and abdominal growths averaged about 2 to 4 cm. in their greatest diameter. The largest found in the series were two masses 4 to 6 cm. in diameter in the anterior mediastinum in case D 1829, and an oval mass 15 cm. in its greatest diameter in the anterior mediastinum of case D 1341. Additional smaller growths in other anatomic sites were found in both of these animals. Nodules were noted on the

Fig. 1, 2, and 3—Lesions found in the heart (incised), intercostal nerves, and brachial plexus of animal D 1394. Photomicrographs of portions of the neoplasm in the brachial plexus and heart are shown in figures 8 and 11, respectively. The microscopic appearance of a portion of one of the nodular growths found adjacent to lymph nodes in the same animal is illustrated in figure 7. Twelve nodules similar to those seen in figure 2 were noted along the intercostal nerves in this animal. Arrows point to three of these nodules in figure 2.

ventral surface of the vertebrae in a few cases where there was extensive involvement of the intercostal nerves. In D 1647, two nodules, about 3 cm. in their greatest diameter, were found on the ventral surface of two thoracic vertebrae in addition to lesions in the intercostal nerves and mediastinum, but these nodules seemed closely related to the proximal end of the intercostal nerves and could not be given an extradural origin. These findings support the observations in man,[1,11,14] that the Schwann's cell tumors of the mediastinal and retroperitoneal regions usually attain a greater size than other similar tumors of peripheral nerves.

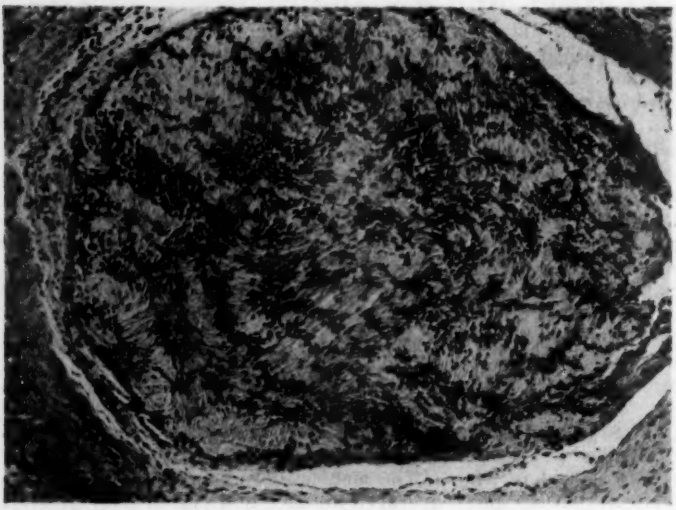

Fig. 4—An encapsulated growth composed of Antoni type-A tissue with distinct formation of Verocay bodies was described as one of six fibrous nodules not over 10 mm. in their greatest diameter found in the heart. No other lesions were noted in this 6-year-old cow (D121). Hematoxylin-eosin. x 90.

MICROSCOPIC DATA

In all of the neoplasms included as schwannomas in this report, areas were found in one or more of the lesions where the tumor cells were elongated and spindle-shaped with oval or long cylindrical nuclei. Distinct palisading of the nuclei was found in each tumor (fig. 4, 5), and

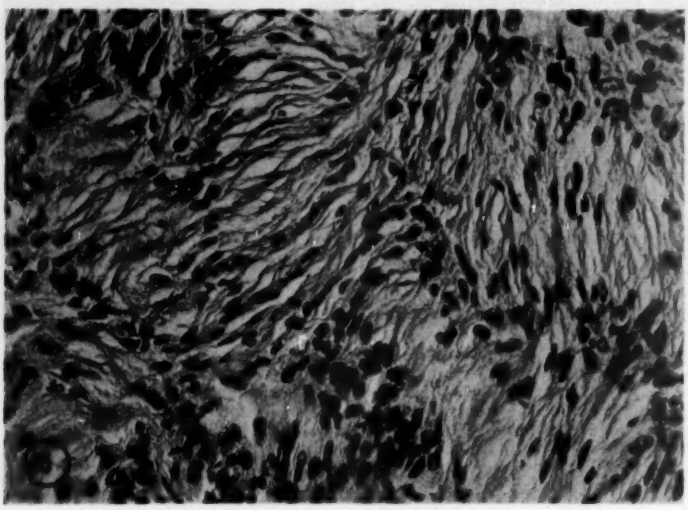

Fig. 5—Schwann's cells with palisading nuclei and the characteristic whorled appearance of Antoni type-A tissue. Note the parallel arrangement of intercellular fibers between Schwann's cells. Intercostal nerve case 14675. Hematoxylin-eosin. x 375.

equally as characteristic was the alignment of cells in interlacing fasciculi and whorls. Intercellular fibers similar to a fine reticulum or, in the more solid or compactly

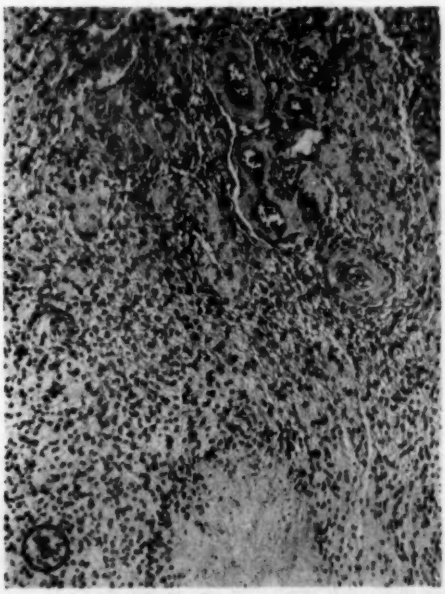

Fig. 6—A heart lesion composed primarily of loosely arranged Schwann's cells similar to those shown under higher magnification in figure 11 but also containing necrotic foci and blood vessels with thick collagen sheaths. A transition to Antoni type-A tissue was found in only one of many sections of this solitary growth (case D 345) in the myocardium described as being as large as a baseball. Hematoxylin-eosin. x 90.

organized tumor, to connective tissue collagen were present. The orderly arrangement in bundles of parallel cells and intercellular fibers and the alternation of fibrous and nuclear zones resemble Antoni type-A tissue or the fasciculated structure of the human neurilemmoma (fig. 4, 5). The whorling of these regimented areas into Verocay bodies occurred in each case (fig. 4, 7, 8) but the suggestion of bizarre tactile corpuscles (fig. 9) resembling Wagner Meissner's corpusles, which have been so clearly depicted by Masson,[8] was more consistently noted in the smaller encapsulated growths. In many of the tumors, the whorling and palisading were not seen in portions of a single lesion or were entirely absent from one or more growths when the lesions were multiple. Palisading of nuclei was also observed in some fibrous and smooth muscle tumors in our series of animal neoplasms and these tumors had to be differentiated from true schwannomas. The tendency to regard connective tissue tumors as schwannomas on the basis of nuclear palisading alone may lead to confusion in diagnosis. Similar difficulties are encountered in the differential diagnosis of connective tissue tumors of man. Areas were occasionally found in the characteristically solid, encapsulated growths composed of the typical Antoni type-A tissue already mentioned in this paragraph in which necrotic and sometimes hemorrhagic centers were present in a meshwork of fine reticular fibers and stellate and spindle-shaped Schwann's cells. Many small vacuoles were present in the intercellular spaces (fig. 6). The vacuoles appeared clear or were partially filled with a hematoxylin-staining mucinoid material. These areas of degeneration were often quite vascular and thick collagen sheaths were present around many of the blood vessels (fig.

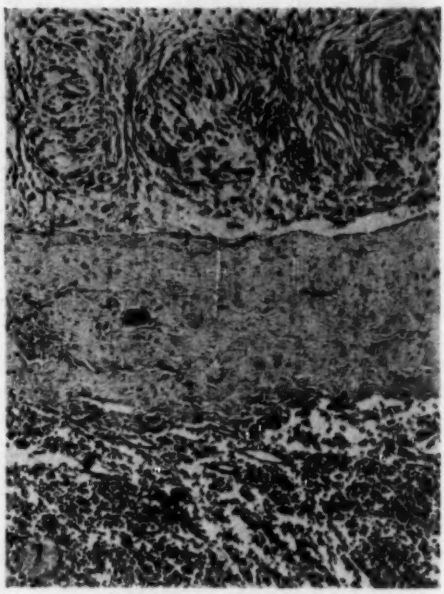

Fig. 7—Antoni type-A tissue reflected on the capsule of a bronchial lymph node. The nerve of origin of the growth (case D 1394) could not be demonstrated. The neoplasm had not penetrated the capsule and was not found in the lymphoid tissue of the node. Hematoxylin-eosin. x 90.

6). The tendency to form large cystic spaces as is described in the closely related Antoni type-B tissue of the human neurilemmoma was not observed in our cases.

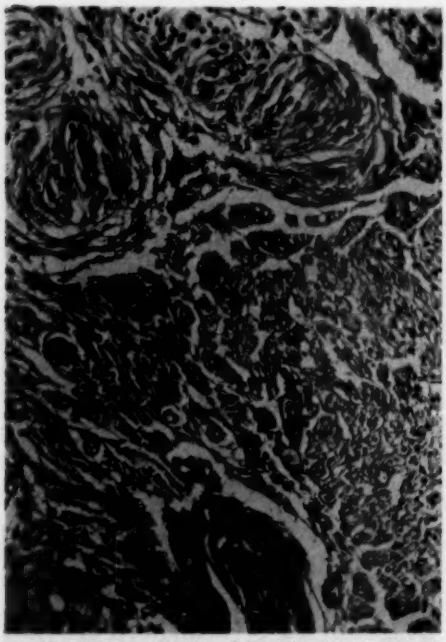

Fig. 8—Verocay bodies almost in direct apposition to the cells and fibers of a nerve of the brachial plexus. Usually, and in most of the lesions in this case (1394), such Antoni type-A tissue is well encapsulated. Hematoxylin-eosin. x 175.

Significantly, a third pattern, found as a nonencapsulated or slightly encapsulated, rather fibrous growth or as a component of the encapsulated neoplastic tissue described in part in the preceding paragraph, was represented by a more diffuse and haphazard arrangement of Schwann's cells, connective tissue elements, and isolated neurites (the neurites were probably sprouts from damaged nerves and should not be considered as having neoplastic properties). In some tumors considered to be more anaplastic, these cells were found arranged in cordlike projections when a loose stroma was present in the adjacent tissues (fig. 10). The resemblance of this loosely textured neoplastic tissue to the lesions of multiple neurofibromatosis of man is acknowledged. However, these lesions, particularly those in the heart (fig. 11) and mediastinum (fig. 10) were consistently more cellular and the nuclei of the tumor cells more oval, bizarre, and hyperchromatic than in the typical neurofibroma of man. Mitotic figures are not demonstrable in these growths. We have no cases in which lesions could be demonstrated in relation to the nerves of the skin. It is only fair to state that some of the plexiform growths of the brachial plexus were indistinguishable from human neurofibromas (fig. 14). Godwin[3] recently pointed out the rarity of encapsulated neurilemmomas of the brachial plexus in man and we have no examples of such solitary lesions as he described in our bovine material.

DISCUSSION

In most animals, excluding lesions found in the heart and mediastinum, it was demonstrated that the suspected tumor either

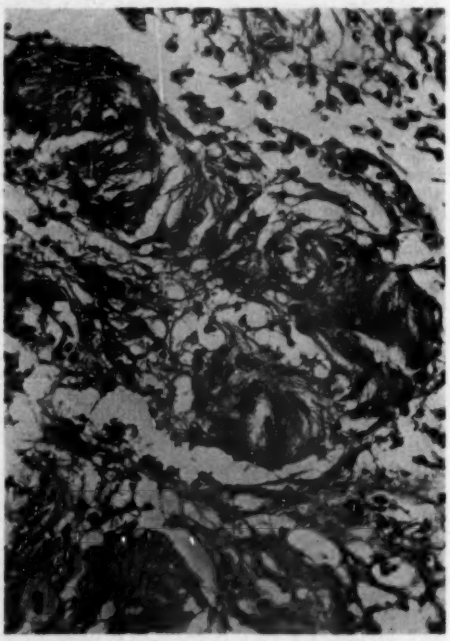

Fig. 9—A rather unusual arrangement of Antoni type-A tissue into structures resembling large giant cells or bizarre tactile corpuscles. In other areas of this lesion of the intercostal nerve (case D 1647), typical Verocay bodies and Antoni type-B tissue were found. Hematoxylin-eosin. x 250.

partially replaced, or was continuous with portions of one or more nerves or ganglia, or grew as tortuous proliferations extending inside of nerve sheaths. The inclusion of individual nerve cells or bundles within many of the tumors (fig. 12, 13) suggested that the nerve had been diffusely expanded by the growth. However, it should be pointed out that the presence of nerve fibers is an incriminating but not a safe criterion of diagnosis by itself, as other sarcomas surround and infiltrate nerves in the tissues where they are growing. It was difficult not to include some of the schwannomas with the malignant schwannomas, particularly the plexiform growths in which many bizarre and hyperchromatic cells were seen. Only 1 tumor of the series (D 1794) was considered to be a malignant schwannoma. It exhibited a marked tendency to appear in wild and variable patterns and to infiltrate tissues, particularly the bronchial and portal lymph nodes where the tumor had replaced the majority of the nodes. Origin from nerve sheaths could not be demonstrated in the lymph nodes so we must regard these lesions as true metastases. Nuclear regimentation, fasciculation, and other structural features consistent with a schwannoma were found in some but not all of the lesions in this case. The relation to lymph nodes can be confusing as encapsulated neoplastic tissue was seen involving a portion of a nerve which was adjacent to a lymph node in 2 other cases (14675 and D 1647) considered to be benign. It illustrates the caution which should be exercised in diagnosing lesions as metastases in this disease without first considering the possibility of a multicentric origin. In addition, involvement of branches of nerves in the parenchyma of visceral organs in some of our animals were demonstrated where there was marked involvement of the larger intrathoracic and intra-abdominal nerves, ganglia, and plexuses. Such lesions were found in the heart, liver, esophagus, and pancreas of case D 155.

The gross appearance and location of lesions of multiple schwannomas at the time of autopsy is usually suggestive of the

Fig. 10—Outgrowths of Schwann's cells in tufts into mediastinal tissues. Portal and bronchial lymph node metastases were present in this case (1794). Hematoxylin-eosin. x 175.

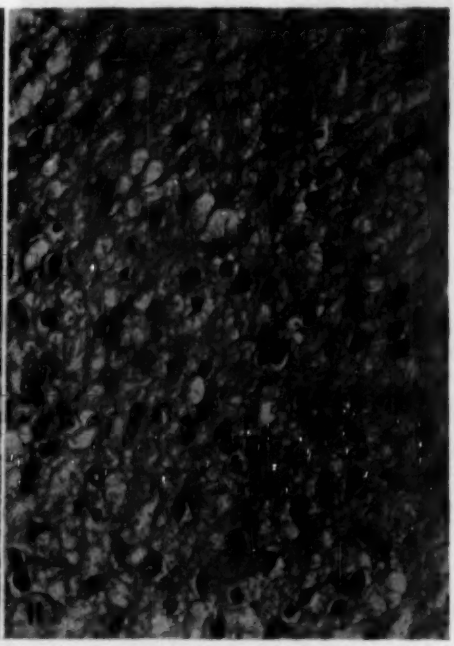

Fig. 11—Schwann's cells with oval to fusiform nuclei in a meshwork of fine reticular fibers containing many small cyst-like spaces (heart case D 1394). Hematoxylin-eosin. x 415.

disease. Nevertheless, the great variation in microscopic appearance of individual lesions, and particularly those found only in the heart, impresses one with the necessity of selecting blocks of tissue from several areas of a suspected Schwann's cell tumor if one is to make a proper differentiation from various benign and malignant mesenchymal neoplasms. The difficulties in establishing a diagnosis if lesions are confined to the heart should be emphasized. In a few instances, individual or small groups of nerve fibers were found within the cardiac growths. It was impossible to ascertain from the available tissue whether these were innervations of neoplastic cells, the normal innervations of the tissue before it was replaced by the neoplasm, or the actual fibers from which the tumor arose. Determination of a point of origin from these nerve endings was further complicated because about one-half of the tumors arising in the heart were only partially or poorly encapsulated and in most instances could be found infiltrating the adjacent musculature. However, one must assume on the basis of our present knowledge and on the anatomic relation of the tumor to nerve in other sites that the true schwannoma of the heart arose from one of the cardiac nerves or its branches. Many of the tumors were noted in the myocardium just beneath the epicardium (fig. 1) and one was found projecting from the surface of the pericardium (fig. 16, 17).

Fig. 12—Ganglion cells of the cervicothoracic ganglion surrounded by proliferating Schwann's cells. The involved ganglion (case D 1829) was about the size of a hen's egg and was distinctly encapsulated. Hematoxylin-eosin. x 500.

In table 1, it is of interest that the heart had the highest incidence of this tumor in the arbitrary groups of locations listed and that in 6 animals, lesions were found only in the heart. Due to the thorough examination of the heart in meat inspection for tapeworm cysts, it is likely that a higher percentage of cases involving these organs would be found than in the brachial plexus or musculature, where great care has to be taken not to disfigure areas from which the commercial cuts of meat are derived until there is adequate justification to suspect that lesions of a definite disease may be found in those tissues.

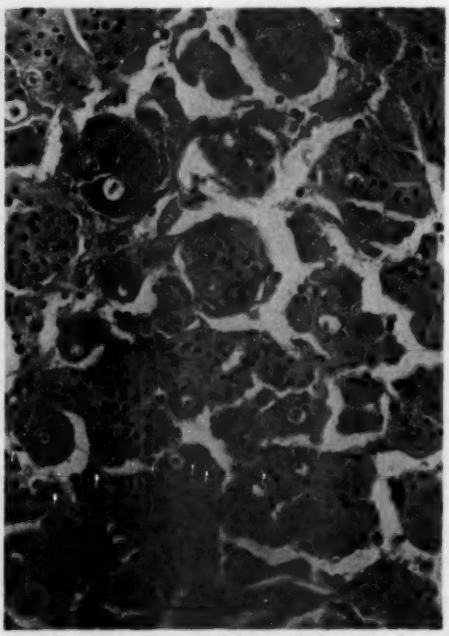

Fig. 13—Proliferating Schwann's cells within a nerve of the brachial plexus. Figure 14 shows involvement of the perineurium of the same nerve (case 17518). Hematoxylin-eosin. x 240.

However, even if solitary or multiple tumors of the brachial or lumbosacral plexuses, their nerves or other nerves of the musculature were overlooked, it would add little information to this paper except to strengthen the observation that lesions are found involving nerves in various anatomic sites. A statistical evaluation of this tumor in regard to either incidence or location would therefore be difficult to obtain. In fact, the observations by the European investigators early in this century that the brachial plexus was more commonly involved is probably correct.

It will also be noted in table 1 that the average age of the cattle included in this report would be 4 to 6 years of age. Three of our affected animals are listed at 2 years. Joest,[7] in Germany, observed the tumor in calves. There is no evidence to dispute a similarity of the growth pattern in regard to age to that of von Reckling-

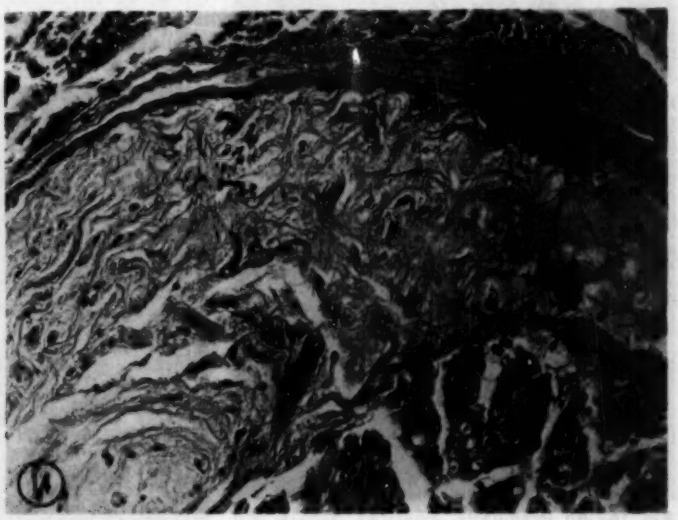

Fig. 14—Loosely arranged Schwann's cells separate the perineurium from epineurium of a nerve of the brachial plexus (case 17518). Hematoxylin-eosin. x 175.

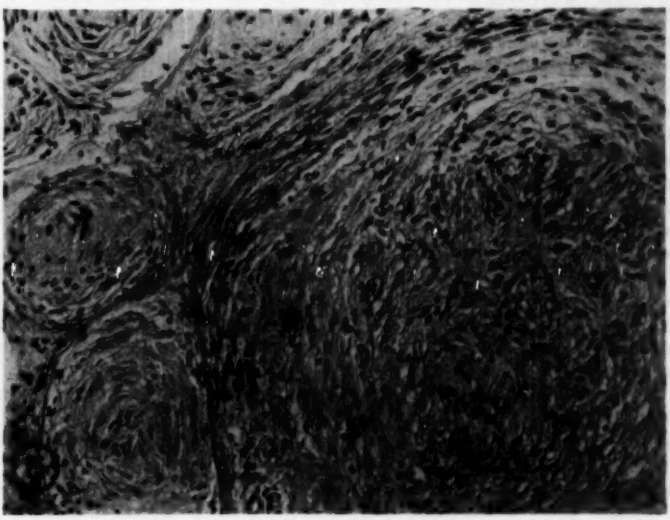

Fig. 15 — Note the distinct whorling of Schwann's cells. Tumor of the intercostal nerve of a 10-month-old pig (case 15873). Hematoxylin-eosin. x 175.

Fig. 16—An irregularly, lobulated, fibrous, and slightly mucinous Schwann's cell tumor arising in the myocardium and pericardium of a white-faced Hereford cow (case D 2231).

hausen's disease in man, where the lesions generally appear first in childhood or adolescence and may become quiescent at full maturity, or to the solitary neurilemmoma of man which may arise at any age. In von Recklinghausen's disease, it is not uncommon for neoplasms, at any time, to enlarge and progress to a fatal termination. The variation in age of the affected cattle would support the view that most of the tumors are relatively benign. It is probable that many of the animals under normal conditions of husbandry would have died from other causes before displaying symptoms which would lead a clinician to suspect a neoplastic disease.

The rarity of multiple schwannomas in other species of domesticated animals is evident in our material. We do not have examples of this neoplasm in sheep, goats, or horses and only 1 case in swine (15873).

Growths were noted to involve many of the intercostal nerves of a 10-month-old barrow which had been sent to slaughter. All of these tumors were distinctly encapsulated and typical Verocay bodies, whorls, fascicles, and palisading of nuclei were easily found. The whorling in some of the lesions was quite pronounced (fig. 15). Other than the extreme whorling, the lesions closely resembled the cases in cattle in which the intercostal nerves were involved.

Fig. 17—The two larger tissues are sections of the tumor shown in figure 16 and the third represents a second independent and more mucinous growth of Schwann's cells (case D 2231).

SUMMARY

Twenty-five cases of multiple schwannomas in cattle and 1 case in a hog are presented in which the diagnosis was confirmed by gross and microscopic examination of tissues.

The tumor is usually benign. In only 1 animal were definite metastases demonstrated. The neoplasm may be found in animals of all age groups.

The heart, brachial plexus, and intercostal nerves were the most common anatomic sites of the tumor. Skin lesions similar to that seen in neurofibromatosis (von Recklinghausen's disease) in man were not encountered.

In most instances, except where lesions

involved the heart or mediastinum, the tumor was found adjacent to, or incorporated in, one or more nerves, ganglia, or plexuses.

The multicentric development of many Schwann's cell tumors in cattle is suggested by the findings of this study.

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High-Level Aureomycin Dosage in Anaplasmosis

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WORK with some of the antibiotics in treatment of anaplasmosis shows such promise that this laboratory has continued its research with aureomycin. Aureomycin used by Foote *et al.*[1] in the recommended dosage had no effect on the course of acute anaplasmosis. Later, however, Miller and his co-workers [2] demonstrated that comparatively small doses of aureomycin and terramycin used in splenectomized calves and adult cattle inhibited the production of Anaplasma bodies in the erythrocytes. When Foote and Wulf [3] used large doses of aureomycin, up to 47.5 Gm., in both acute and carrier cases of anaplasmosis, the antibiotic was much more effective. The present authors have previously shown [4] that even a large dose of aureomycin did not permanently destroy carrier infection but did greatly modify the Anaplasma organism, at least temporarily. This article reports additional studies using various large doses of aureomycin in both acute and carrier cases of anaplasmosis.

METHODS

The cattle inoculated to test the carrier state of treated cattle were either splenectomized three weeks or more before inoculation or, in unsplenectomized animals, were exposed later to known carrier blood. Blood samples were routinely drawn every second day and occasionally each day. The anaplasmosis-infected cattle were treated intravenously with aureomycin in sterile distilled water at the rate of 5 Gm. per 250 ml. Blood samples were taken from the jugular vein into bleeding tubes containing 2 drops of saturated solution of sodium citrate. Slides made from these samples were stained with Giemsa's blood stain. The amount of blood used to infect the test animals varied from 3 to 5 ml. All injections of inoculum into test animals were made subcutaneously. Animals were not assumed to be negative to Anaplasma infection following inoculation until they had passed the sixty-fifth day without showing evidence of anaplasmosis.

EXPERIMENTAL PROCEDURE AND RESULTS

Six adult cows infected with anaplasmosis, 2 in the carrier state, 2 in convalescence, and 2 in the acute phase of the disease, were treated with various amounts of aureomycin to determine more fully the value of aureomycin in the treatment of anaplasmosis. The results of this study are summarized in tables 1 and 2.

TREATMENT OF CARRIER CATTLE

Animal AH.—A 4-year-old purebred Hereford cow, weighing 1,200 lb., had contracted a field case of anaplasmosis in September, 1951. Her blood produced anaplasmosis in 2 inoculated cows in January, 1952. She was treated with 50 Gm. of aureomycin in 10-Gm. doses twelve hours apart, starting on March 6, 1952. This treatment was repeated thirty-three days later starting on April 10, 1952.

Between the first and second treatments, 2 splenectomized calves (204 and 200) were inoculated with blood from AH. Both remained negative to anaplasmosis.

Following the second treatment, 6 cows (206 splenectomized; 186, 208, 231, 245, and 242) were inoculated with blood from cow AH at intervals ranging from three days to 263 days after completion of the second treatment. None of these animals developed anaplasmosis.

Animal 139.—This 600-lb. cow was used in work previously reported by the authors [4] in which she had acquired a mild modified anaplasmosis infection from cow 411 in October, 1951. On Dec. 31, 1951, she was inoculated with a virulent Anaplasma organism and on subsequent subinoculation caused typical anaplasmosis in the recipient animals.

Starting on April 10, 1952, animal 139 was given 15 Gm. of aureomycin in 5-Gm. doses twelve hours apart. No second treatment was given.

Three cows (207 splenectomized; 145, and 225) were inoculated with blood from cow 139 at intervals ranging from three to 137 days following completion of the aureomycin treatment. All of these cows became sick, showing clinical symptoms of anaplasmosis.

TREATMENT OF CONVALESCENT CATTLE

Animal 142.—This 600-lb. yearling Hereford became infected with anaplasmosis in late January, 1952. The peak percentage of erythrocytes infected was 25 per cent and the lowest r.b.c.

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count was 2,620,000. When aureomycin treatment was started on March 6, 1952, the r.b.c. count was 6,270,000. This animal was given 25 Gm. of aureomycin in 5-Gm. doses twelve hours apart. No second treatment was given.

TABLE 1—Treatment of Anaplasmosis with Aureomycin

Animal (No.)	Age (yr.)	State of Anapl. at 1st treat. r.b.c. (millions)	State of Anapl. at 1st treat. r.b.c. (% infect.)	Treatment 1 Amt. (Gm.)	Treatment 2 Days after treatment	Treatment 2 Amt. (Gm.)
AH	4	8.56	0.0	50.0	33	50
139	1	9.35	0.0	15.0	....	....
143	1	6.27	0.0	25.0	....	....
McG	4	4.50	0.05	50.0	26	50
208	2	3.82	3.0	37.5	....	....
Zato	2	2.65	1.0	30.0	22	30

Two splenectomized calves (203 and 201) were inoculated from cow 142 on the second and thirty-sixth days following completion of the aureomycin treatment. Both calves developed anaplasmosis.

Animal McGuire.—This 4-year-old, 900-lb. Guernsey cow was obtained from a local ranch on Sept. 5, 1952. She then had a r.b.c. count of 2,240,000 and showed a few Anaplasma bodies in the erythrocytes. Starting on Oct. 13, 1952, this cow was given 50 Gm. of aureomycin in 10-Gm. doses twelve hours apart. This treatment was repeated twenty-six days later.

Animal 249 was inoculated with blood from McGuire on the seventeenth day following McGuire's first treatment. This animal developed anaplasmosis. Two cows (254 and 241) were inoculated with blood from McGuire following the second treatment. Number 254, inoculated on the seventh day, did not become infected with anaplasmosis; cow 241, inoculated on the fifty-first day, developed anaplasmosis.

TREATMENT OF ACUTE ANAPLASMOSIS IN CATTLE

Animal 208.—This 2-year-old, 700-lb. Jersey heifer was used in July, 1952, to test the carrier infection of AH and remained negative. In September, 1952, she was inoculated with known carrier blood to check her susceptibility to anaplasmosis. She became infected showing a minimum of 3,600,000 r.b.c. and a maximum of 12 per cent Anaplasma-infected erythrocytes. At the time treatment with aureomycin was started on Oct. 16, 1952, cow 208 showed 3,820,000 r.b.c. and 3.3 per cent infected erythrocytes. Aureomycin treatment consisted of 37.5 Gm. given in 7.5-Gm. doses twelve hours apart. This treatment was not repeated.

Two cows (243 and 246) were inoculated with blood from cow 208. Cow 243, inoculated on the sixteenth day after the aureomycin treatment, remained negative. Animal 246, inoculated on the seventy-fourth day following the treatment, developed anaplasmosis.

Animal Zato.—This purebred 1,100-lb. Hereford bull contracted a field case of anaplasmosis in October, 1952. He was given 30 Gm. of aureomycin in 10-Gm. doses twelve hours apart, when the r.b.c. count was 2,650,000 and the percentage of infected erythrocytes was 1 per cent. This was the lowest r.b.c. count but was past the peak of the number of Anaplasma-infected erythrocytes. The aureomycin treatment was repeated on the twenty-second day.

One yearling heifer (240) was inoculated with blood from Zato on the twenty-first day following the first treatment. She did not become infected with anaplasmosis.

Two animals (247 and 250) were inoculated with blood from this bull following the second treatment. Animal 247, inoculated on the fourth day after treatment, remained negative. Animal 250, inoculated on the forty-sixth day after treatment, became infected with anaplasmosis.

In an attempt to clarify the length of the negative phase, 2 Anaplasma-infected splenectomized calves were treated with 7.5 Gm. and 12.5 Gm. of aureomycin. Following this treatment, there was a period of nineteen to thirty-three days when no Anaplasma bodies could be found in the erythrocytes and the r.b.c. count increased from 3,000,000 to 8,500,000. After this negative period, Anaplasma bodies again appeared, the blood count dropped, and the animals continued in the recurrent cycle typical of splenectomized calves.

DISCUSSION

The thought at the beginning of this experiment was to use approximately the same dosage of aureomycin as that used on cow 411 reported in the previous study,[4] but to repeat it at about thirty days. This time interval was suggested by the fact that cow 411 was negative for that period before she again became positive. When this method was tried on cow AH she remained negative to the time of this writing (296 days following the first treatment), and it is possible that she is free of carrier infection.

Unsuccessful attempts were made to duplicate the results obtained with AH and with other dosage schedules on animals in various stages of anaplasmosis.

Cow McGuire, although smaller than AH, was given the same dosage of aureomycin; however, McGuire was negative for only a short time following the second treatment. This is similar to the result obtained with cow 411 when only one treat-

ment was used. The infection transmitted by McGuire was fully virulent as contrasted to the seemingly less virulent infection transmitted by 411 when she became positive following treatment.

In this study, we have found little evidence of modification of the virulence of the causative organism of anaplasmosis. Either there was no infection or the case was typical of the usual cases of anaplasmosis.

We are unable to advance the reason for the failure of aureomycin to produce similar results in McGuire and AH. There were, of course, many factors of possible difference between the 2 animals. There may have been a difference in the resistance of the two organisms to aureomycin or, if the organism has a cyclic life history, there may be a period during which the organism is less resistant to the drug; these and a great many other possible factors must remain hypothetical until we have a more complete basic knowledge of the causative organism of anaplasmosis.

Aureomycin was more effective in the acute stage of anaplasmosis (208 and Zato) than it was on the convalescent case, McGuire. The length of the negative phase was prolonged in the 2 animals treated in the acute stage.

When the total dose of aureomycin was less than 30 Gm., in this series of experimental cows, there was little apparent effect on the Anaplasma organism. This is demonstrated by the 15-Gm. and 25-Gm. doses given animals 139 and 142. Both transmitted anaplasmosis by subinoculation within three days after treatment. When 30 Gm. or more of aureomycin was used, there followed a period when anaplasmosis was not transmitted by subinoculation. There is a question as to the presence of such a negative phase in McGuire following the first treatment of 50 Gm. of aureomycin. Due to experimental difficulties, no subinoculation was made from her until the seventeenth day following the first treatment. She may or may not have passed through a negative phase before the positive subinoculation was made on the seventeenth day. Likewise, the adult carrier cow, treated by Miller *et al.*[2] with 2.5 Gm. of aureomycin daily for twenty days, transmitted anaplasmosis on the tenth day (after receiving 25 Gm. of aureomycin) but did not transmit anaplasmosis on the twentieth day (after receiving 50 Gm. of aureomycin). These authors do not state whether the cow later resumed transmitting anaplasmosis.

These data indicate that less than 30 Gm. of aureomycin will not produce a negative phase in adult carrier cattle. Also,

TABLE 2—Tests for Carrier Infection of Anaplasmosis

		After 1st treatment				After 2nd treatment			
				Results				Results	
Animal tested	Animal inoculated	Days	Incubation days	r.b.c (millions)*	r.b.c.** (% infect.)	Days	Incubation days	r.b.c (millions)*	r.b.c.** (% infect.)
	204	2	....	.....	....				
	200	33	....	.....	....				
	206					3	....	.....	....
	186					37	....	.....	....
AH	208					81	....	.....	....
	231					181	....	.....	....
	245					244	....	.....	....
	242					263	....	.....	....
	207	3	48	2.25	14				
139	145	34	25	2.17	25				
	225	137	20	3.06	14				
142	203	2	45	2.49	17				
	201	36	41	1.94	25				
	249	17	27	2.63	22				
McG	254					7	....	.....	....
	241					51	18	4.85	3
208	243	16	....	.....	....				
	246	74	23	5.20	8				
	240	21	....	.....	....				
Zato	247					4	....	.....	....
	250					46	19	5.16	2

* Minimum count of red blood cells reached during infection; ** maximum percentage of red blood cells infected during infection.

the total amount of aureomycin used seems to be the determining factor in its effect on the anaplasma organism, whether the total amount is given in three days, repeated again in thirty days, or, as used by Miller, given over a period of twenty consecutive days. If a few large doses of aureomycin will produce the same results as many repeated smaller doses, that treatment will be a definite advantage to the practitioner and those handling range cattle.

Due to the lack of test animals the exact length of the negative phase, following treatment, could not be determined, but it was bracketed by the time the last test animal remained negative and the first one became positive. These data indicate that the negative phase is probably from sixteen to forty-six days following adequate treatment. It is interesting to note that this negative phase, in so far as we know it, coincides quite closely with incubation periods in anaplasmosis. The authors feel that the presence and length of this negative phase is mainly important in that it may show a way to increase our knowledge of the nature of the Anaplasma organism. It may eventually lead to consistent destruction of carrier infection but as yet the cost of the aureomycin apparently necessary makes it prohibitive.

The authors suggested, in a previous report [4] a theory to account for the negative phase produced by aureomycin, when used in cattle infected with anaplasmosis. In this theory, a cyclic life history with at least two stages of the Anaplasma organism was postulated. One stage in the peripheral blood is infectious and susceptible to aureomycin; the second, apparently located in some other tissue of the body and probably intracellular, is resistant to aureomycin and either is not infectious or is not included in the inoculum taken from the peripheral blood stream. While this theory is in agreement with many of the known facts concerning anaplasmosis, and can be used to explain the difficulty experienced in culturing the organism artificially, it does not explain the shortened or nonexistent incubation periods when extremely large amounts of inoculum are used.

Another explanation for the negative phase may be found in the action of aureomycin on the Anaplasma organism. Large doses of aureomycin may interfere sufficiently with the metabolism of the organism to reduce its virulence and ability to reproduce but not enough to kill it or allow the body to destroy it. Then, following a period of recovery from the effects of the aureomycin, possibly in succeeding generations of the organism, a fully virulent organism is again produced. This theory, however, does not explain why the aureomycin-injured Anaplasma organism when transmitted does not eventually cause anaplasmosis in the recipient animal.

It may be that neither of these theories is completely or even partially correct, but they are presented here as stimuli for additional work on the basic nature of the Anaplasma organism.

SUMMARY

Six anaplasmosis-infected cows were subjected to various repeated doses of aureomycin. One adult carrier cow has not transmitted anaplasmosis on subinoculation for 296 days following the first treatment with aureomycin. Another similar carrier cow, given the same dosage of aureomycin, had only a short period when she did not transmit anaplasmosis on subinoculation. Cows receiving less than 30 Gm. of aureomycin transmitted anaplasmosis whenever tested. Cattle receiving 30 Gm. or more of aureomycin passed through a negative phase when anaplasmosis was not transmitted by subinoculation.

Two possible theoretical explanations are presented to explain the negative phase produced by the treatment of anaplasmosis-infected cattle with aureomycin.

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The Action of Hypochlorite and Other Disinfectants on Micrococci with and Without Milk

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HYPOCHLORITE COMPOUNDS have been employed widely for dairy disinfection.[1]

In a previously reported study of an infected herd, the authors found that disinfection by two successive dippings in calcium hypochlorite solution did not free teat cups of *Micrococcus pyogenes*.[2] In that investigation, coagulase positive micrococci in naturally contaminated teat cups appeared to resist disinfection to a greater degree than coagulase negative micrococci.

Chlorine solutions in the absence of organic matter were shown by Tonney, *et al.*[3] to be highly germicidal for *Staphylococcus aureus*. They found that all of the five strains tested were killed by 0.15 parts per million (p.p.m.) within one minute. Organic matter greatly reduces the bactericidal action of hypochlorites[4] and, also, of quaternary ammonium compounds.[4] The latter compounds have been employed extensively in dairy disinfection in recent years. They appear to have such a high bacteriostatic and clumping effect that their exact evaluation by the standard Food and Drug Administration (F.D.A.) method of comparison with phenol was not considered reliable.[5, 6] The quaternaries have been found very susceptible to the inactivating effect of 10 per cent defibrinated horse blood,[7] 20 per cent cow manure, 50 per cent evaporated milk, or 50 per cent defibrinated bovine blood.[4]

Better methods of disinfecting teat cups are needed. To obtain further fundamental data, a comparison was made of the *in vitro* disinfecting effect of sodium and calcium hypochlorite, phenol, and the quaternary ammonium compound, rocecal,® on coagulase positive and coagulase negative cultures of *M. pyogenes*. The effect of the presence of milk during disinfection was also tested.

MATERIALS AND METHODS

The disinfection tests were all made according to a modified F.D.A. method.[8] The medium was:

Bacto-beef extract	6 Gm.
Bacto-peptone	10 Gm.
Sodium chloride	5 Gm.
Distilled water	1,000 ml.

The ingredients were boiled to facilitate solution, cooled, adjusted to pH 6.8 with bromthymol blue, distributed in 10-ml. amounts, and sterilized under steam pressure at 120 C. for forty minutes. The standard phenol solution was 5 per cent which was diluted further in water to make total final dilutions of 1:60, 1:70, 1:80, and 1.90. Sodium and calcium hypochlorite were dissolved to make dilutions of about 1:5,000. These solutions were then analyzed for active chlorine by the method described by McCulloch.[9] The result of such titration in different experiments varied from 155 to 212 p.p.m. In a similar manner, 1:10,000 and 1:15,000 dilutions of the hypochlorites were prepared and analyzed for active chlorine. The results varied from 90 to 105 p.p.m. for the 1:10,000 dilution and from 56 to 70 p.p.m. for the 1:15,000 dilution. Higher concentrations were also prepared for certain experiments.

Solutions of roccal were prepared by dilution of the commercial 10 per cent concentration to give final concentrations of 1:2,500, 1:5,000, and 1:10,000. In some of the experiments, 1, 2, or 3 per cent reconstituted skimmed milk was mixed with the disinfecting solutions. The effect of the addition of skimmed milk on the concentration of chlorine in disinfectant solutions was tested by titration for chlorine at fifteen-minute intervals for an hour after mixture of milk and disinfectant.

Individual isolations of micrococci, hereafter called strains, were obtained from the milk and from the environment of the dairy cows and held at 5 C. on Difco blood agar base slants. The appearance of the micrococci on blood agar and their coagulase reactions were established as previously described.[2] No regard was paid to the pigment production by these strains. The strains were inoculated into the broth medium and allowed to

TABLE 1—Effect of Skimmed Milk on the Titratable Chlorine in a Disinfecting Solution* of Sodium Hypochlorite at Various Time Intervals

Milk added (%)	Time in minutes				
	0	15-25	30-40	45-55	60-70
0.0	236	220	231	217	226
3.0	146	113	113	97	96
2.0	123	104	100	97	56
1.0	155	99	95	85	83
0.5	182	127	114	104	102
0.25	203	165	149	142	130

* The initial concentration was 236 parts per million and the figures reported are in parts per million of available chlorine.

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grow at 37 C. for twenty-four hours. They were then transferred to fresh medium for another 24-hour period. The cultures were mixed thoroughly and 0.5-ml. amounts were transferred to each 5-ml.

TABLE 2—Standard Experiment Performed to Determine the Resistance of Strain 42 (Coagulase Positive) Against Phenol and Hypochlorite Disinfectants*

Disinfectant	Concentration	Growth after action in minutes		
		5	10	15
Phenol	1:60	0	0	0
	1:70	+	0	0
	1:80	+	+	0
	1:90	+	+	+
Calcium hypochlorite	1:5,000 (191 p.p.m.)	+	+	0
	1:10,000 (99 p.p.m.)	+	+	+
	1:15,000 (57 p.p.m.)	+	+	+
Sodium hypochlorite	1:5,000 (144 p.p.m.)	+	0	0
	1:10,000 (85 p.p.m.)	+	+	+
	1:15,000 (71 p.p.m.)	+	+	+

* Viability in the disinfectant solution was checked after five, ten, and fifteen minutes.
0 — no growth; + — growth which is an indication of incomplete disinfection at the time and concentration recorded.

volume of disinfectant solution held at 20 C. At five-, ten-, and fifteen-minute intervals a 4-mm. loopful of each of the mixtures of disinfectant and culture was transferred to 10 ml. of the modified F.D.A. broth. The transfers were incubated at 37 C. for forty-eight hours and examined for the presence of growth. When roccal was employed, those subcultures that failed to show growth after forty-eight hours were inoculated with a viable dilute culture to detect the possible presence of inhibiting amounts of the disinfectant. All these cultures grew readily, indicating that bacteriostatic amounts of disinfectant had not been transferred.

In each experiment, phenol dilutions were tested, along with the other disinfectants, to provide a standard. The usual protocol of an experiment on a single culture is given in table 2.

RESULTS

The effect of addition of skimmed milk on the persistence of titratable chlorine is presented in table 1. Milk caused an immediate reduction in titratable chlorine in approximate ratio to the amount added. Subsequently, there was a further reduction so that by the end of an hour the concentration was considerably reduced. These results indicate that the concentration of milk which might accumulate in the disinfectant under farm condition could reduce the amount of available chlorine by one half to three fourths.

A sample protocol of an experiment to determine the effect of disinfectants on *M. pyogenes* is presented in table 2. A concentration of 191 p.p.m. of calcium hypochlorite was about equivalent to phenol in a dilution of 1:80 for this strain. A comparison of the relative resistance of coagulase positive and coagulase negative strains is indicated in table 3. These results, as well as those at five and fifteen minutes which are not included, indicate that coagulase positive strains are approximately as susceptible as coagulase negative cultures to hypochlorites. There were no apparent differences in the susceptibility of these two types of strains to phenol. Sodium hypochlorite was slightly more active than calcium hypochlorite, an effect that may be due to the higher pH of the latter. Roccal under the same conditions killed the ten strains tested in five minutes at a concentration of 1:10,000.

Skimmed milk depressed the disinfecting action of the hypochlorites as shown in table 4. Sodium and calcium hypochlorite reacted similarly to the effects of milk and no strains were killed in 3 per cent milk. Results obtained after five and fifteen minutes indicated also that these small amounts of milk interfered with disinfection to a much greater degree than would be anticipated from the reduction of titratable chlorine alone. Roccal was not inactivated by 1 per cent milk, only slightly so by 2 per cent, and moderately by 3 per cent milk. Phenol action was affected only slightly by 3 per cent milk.

DISCUSSION

The results obtained here with the *in vitro* effect of milk on disinfection help to

TABLE 3—A Comparison of the Resistance of Strains of Coagulase Positive and Coagulase Negative Micrococcus Pyogenes to Sodium and Calcium Hypochlorite Disinfectants Acting for Ten Minutes in Aqueous Solution

Disinfection	Minimum lethal concentration (p.p.m.)	Number of cultures	
		Coagulase (+)	Coagulase (—)
Calcium hypochlorite (78 strains)	>155-202	22	5
	155-202	40	10
	85-100	0	1
	56-70	0	0
Sodium hypochlorite (78 strains)	>155-202	6	1
	155-202	47	7
	85-100	8	4
	42-70	1	4

TABLE 4—Effect of Skimmed Milk on the Disinfecting Action of Sodium and Calcium Hypochlorite and Roccal®

Disinfectant concentration *	Calcium hypochlorite **			Sodium hypochlorite **			Roccal **		
	1:1,250	1:2,500	1:5,000	1:1,250	1:2,500	1:5,000	1:2,500	1:5,000	1:10,000
3.0% milk	0	0	0	0	0	0	10	7	1
2.0% milk	5	0	0	8	1	0	10	10	6
1.0% milk	10	1	0	10	5	0	10	10	10
0.5% milk	10	8	2	10	9	6			

* Ten strains tested at each concentration; ** number of strains killed after ten minutes.

explain the occurrence of *M. pyogenes* in the disinfectant solution used for teat cups as reported previously.[2] The importance of frequent changes of disinfecting solutions is emphasized. In ordinary milking procedures, dipping teat cups in 9 liters of disinfectant may readily result in 1 per cent contamination by milk after use for 20 cows. It appears advisable to replace the solution even more frequently to avoid significant inactivation of the disinfectant.

Coagulase positive strains and coagulase negative strains had similar resistance to the action of hypochlorites. The previously reported results, in which there was a greater survival of the coagulase positive micrococci in teat cups after disinfection, must be explained by factors other than their inherent susceptibility. It is probable that coagulase positive organisms in milk are more often surrounded by leukocytes and fibrin than are coagulase negative micrococci. This organic matter may protect the coagulase positive micrococci against disinfectants.

Results with roccal are too limited to indicate its possible effectiveness as a teat cup disinfectant. The methods used were inadequate to compare quaternaries with other disinfectants. Condemnation of quaternaries as dairy disinfectants should not be based on experiments involving 10 to 50 per cent organic matter, since such contamination would not usually be found in actual practice. Further work to develop a reliable method of disinfection of teat cups seems indicated.

SUMMARY

Coagulase positive strains of *Micrococcus pyogenes* were approximately as susceptible as coagulase negative strains to hypochlorite disinfectants. Three per cent milk in the disinfectant prevented complete killing by hypochlorites whereas smaller amounts of milk had progressively less effect. Phenol and roccal® were affected much less than hypochlorites by the addition of milk.

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A Report of the Screening of Aralen Dihydrochloride for Activity Against *Anaplasma Marginale*

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ARALEN has been reported by workers in Oklahoma [1, 3] to be remarkably effective in the treatment of anaplasmosis.

In February, 1950, Farley *et al.*[1] reported a 100 per cent recovery of 9 mature animals studied under laboratory conditions. They used aralen-dihydrochloride at the rate of 400 to 900 mg., in two doses at 48-hour intervals. In December, 1950, Pearson [3] reported approximately 90 per cent recoveries of 89 cases using aralen-dihydrochloride. Forty-five of these were field cases and 44 were laboratory cases. The dosage, 4.5 to 9.0 Gm., given in Pearson's paper [3] is no doubt a misprint, as the manufacturers [4] reported in June, 1951, that the recommended dosage of aralen-dihydrochloride was 400 to 900 mg., the same as that reported in February, 1951.[1]

Following these reports, aralen-dihydrochloride was used in large quantities in Louisiana for the treatment of anaplasmosis. In some instances, it appeared to help materially; in others it seemed to be singularly ineffective. With this background it was decided to test the drug under experimental conditions in an attempt to evaluate it on some basis other than mortality.

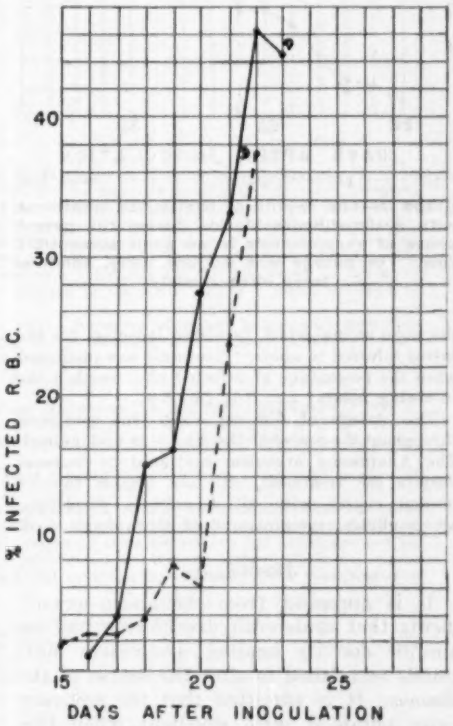
PROCEDURE AND RESULTS

The first step was to determine the effect of the drug against *Anaplasma marginale* by using splenectomized calves in a method previously outlined.[2]

Two groups of 5 splenectomized calves each were inoculated with blood from a common virulent carrier source, the same animal used in previous experiments.[2] Treatment was started and repeated daily after 2 per cent of the r.b.c. were carrying *marginale* bodies. One group was given 5.8 mg. per pound of body weight, this being divided into two equal doses given four hours apart. The second group of calves received 8.7 mg. per pound of body weight, given in three equal doses four hours apart. These dosages were based on 4.5- to 9.0-Gm. dose for mature animals (5.8 and 8.7 mg. per pound of body weight). No activity against the Anaplasma organism could be demonstrated in these tests. With the administration of the higher dosage level, all calves died.

With both the 5.8- and 8.7-mg. dosage on a daily basis (divided as mentioned above) the Anaplasma sp. continued to multiply percentagewise and the erythrocyte infestation pattern was essentially identical to that observed in the companion control calves inoculated from the same source animal. It will be noted that even this lower dosage rate is higher than that recommended by the manufacturer.[4]

At this point, it was evident that the aralen-dihydrochloride showed no activity against the Anaplasma sp. in the concentrations used in the splenectomized calves. However, because of its widespread use in the field, it was decided to re-



———— untreated control; — — — — treated calf.

Graph 1—The results of continuous treatment with aralen-dihydrochloride during the patent phase of anaplasmosis in a splenectomized calf. The dosage was 5.8 mg./lb. daily, administered intravenously in a divided dose four hours apart.

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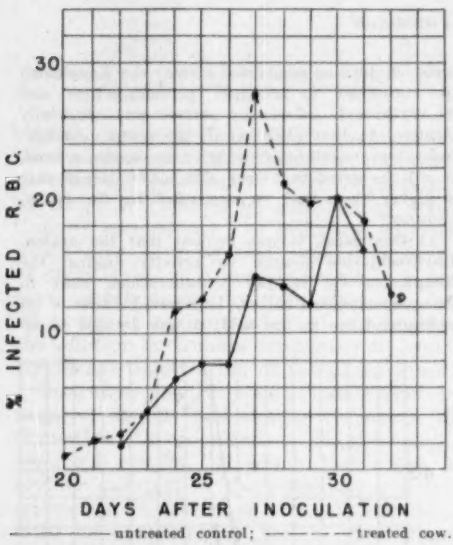
check our results in adult animals. The dosage decided upon was 500 mg. to be administered intravenously once daily. Four adult, susceptible animals were inoculated with carrier blood from



Graph 2—The results of continuous treatment with aralen-dihydrochloride during the patent phase of anaplasmosis in an adult susceptible cow. The dosage was 0.5 Gm. daily, administered intravenously.

the same animal which furnished inoculum for the calves referred to above. Treatment was instituted when the percentage of infected r.b.c. reached the following points: 2.2; 2.6; 2.6; 6.6.

This dosage of 500 mg. daily was continued throughout the course of the disease in each animal. The Anaplasma organism continued to increase despite the treatment, and the disease ran its normal course with all 4 animals developing severe clinical symptoms; 2 of the animals died.

DISCUSSION

It is apparent from the above experiments that aralen-dihydrochloride had no specific activity against *Anaplasma marginale* and failed to alter the course of the disease. It is admitted that the recovery rates obtained were excellent when this drug was used in Oklahoma and left little to be desired, if these results could be attributed to the action of the drug.

The recovery rates in treated cases of anaplasmosis in Oklahoma [1, 3, 4] are much higher than those experienced in Louisiana. A report by Smith and Howell [4] on chemotherapy of 275 cases in Oklahoma indicated quite good results with a wide variety of treatments. In Louisiana, the mortality varies widely from farm to farm and from year to year. We have no records of 90 per cent recovery in either treated or untreated cases.

It would appear that the conditions under which this disease exists in Oklahoma and Louisiana differ materially. No doubt the virulence of the causative organism, type of husbandry generally practiced, care after the animal is treated, and other factors, account for the severity of the disease and mortality. A field survey made in Oklahoma by Smith and Howell [5] showed that only 14.8 per cent of 175 animals had below 4.8 Gm. of hemoglobin per 100 cc. of blood when first seen. The authors considered this to be a level below which survival is doubtful. Of 60 field cases of anaplasmosis in Louisiana, 82 per cent had below 4.8 Gm. per 100 cc. of blood at the time a veterinarian was called.

The following statement from the article by Smith and Howell [4] in October, 1944, seems to be of paramount importance in studying any drug being used to treat anaplasmosis: "Successful treatment depends upon recognition of the disease in the initial stage, and early elimination of the Anaplasma parasite from the blood cells, by some chemotherapeutic agent without damage to the blood forming organs."

The above quotation supports our belief that drugs to be used in the treatment of anaplasmosis must be tested and their effectiveness evaluated against some part of the disease which can be measured. For this reason in our experiments, we studied the effect of the drug upon the multiplication of the Anaplasma organism, one phase of the disease which is quite constant.

SUMMARY

1) Aralen-dihydrochloride at very high dosage levels did not exhibit any activity against *Anaplasma marginale* in splenectomized calves.

2) Aralen-dihydrochloride given to 4 adult, susceptible, inoculated cows, at the rate of 0.5 Gm. intravenously daily, did not alter the course of the disease nor ameliorate the symptoms.

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Studies on Intravenous Administration of Calcium, Potassium, and Magnesium to Dairy Calves
I. Some Biochemical and General Toxic Effects

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DISORDERS of dairy cattle, characterized by changes in levels of several mineral blood constituents, have long been recognized. Milk fever and grass tetany are notable examples. Moreover, the frequent occurrence of these diseases has led to the widespread use of parenterally administered calcium salts as a means of treatment. Magnesium salts have sometimes been incorporated in calcium solutions as an aid in therapy. The administration of potassium salts has had occasional use in the treatment of other diseases. The injection of any of these three cations may produce severe toxic effects and even death. In view of these facts, efforts were made to learn more about the physiological and toxic effects of these salts when injected intravenously in dairy cattle.

METHODS

Seven male and 5 female dairy calves (10 Holstein-Friesians and 2 Guernseys), ranging in weight from 48 to 240 lb., were restrained in special hammock-type stocks[3] and subjected to a series of 29 experiments.

Seven of these experiments consisted of the injection of 6 per cent calcium chloride, two of 10 per cent calcium gluconate and 2 per cent boric acid, eight of 3 per cent to 8 per cent potassium chloride, five of 12 per cent magnesium chloride hexahydrate, two of 1.5 per cent sodium oxalate, three of 25 per cent glucose, and two of calcium chloride and magnesium chloride simultaneously.

These experiments were carried out by cannulating the jugular vein with a polyethylene tube and subjecting the animal to a continuous and nearly constant rate of injection until severe symptoms or death occurred. Magnesium injections were carried to the point of light anesthesia. In most cases, a constant infusion mercury pump was used. Various rates and durations of injections were studied. Fatal total dosages and rates of injections could then be roughly determined for several of the ions. Average values are given in table 1.

Electrocardiograms were taken in all of these experiments.[3] Respiratory movements, phonocardiograms, and right ventricular pressures were also recorded in the majority of the experiments.[5]

Blood samples were collected before the injections, immediately after cessation of the injections, and, in most cases, during the injections and at variable times during the recovery period. Glucose was determined on whole blood specimens; total calcium was determined on serum specimens; magnesium, inorganic phosphate, sodium, and potassium were determined on heparinized plasma specimens collected by means of an oiled 50-ml. syringe, discharged under oil and thence centrifuged. Four milligrams of heparin were added to 50 ml. of blood.

Glucose was determined by a photometric adaptation[19] of the Somogyi method. Total calcium was determined by the Clark-Collip[4] modification of the Kramer-Tisdall method. Magnesium was determined by the method of Heagy.[10] Inorganic phosphate was determined by the Fiske-Subba Row[17] method. Sodium and potassium determinations were made flame photometrically[2,14] on trichloroacetic acid filtrates of plasma, using the internal standard method.

Levels of blood glucose were expressed as milligrams per 100 cc. All of the other blood constituents were expressed as milliequivalents per liter of serum or plasma. Inorganic phosphate was expressed as phosphorus, assuming an equivalence of 1.8 per atom of P.[18]

RESULTS

Table 1 gives the average cumulative doses, durations, and rates of injections for the various experiments. These values are listed with regard to recovery or death of an animal. From this table can be seen the nature of some lethal factors in a given experiment. The total dose, the blood level attained, and the rate of injection all appeared to be factors in causing death. Figures 1, 2, and 3 are graphic representations of the rise in blood levels for all animals given calcium, potassium, and magnesium, respectively.

Calcium Administrations.—Of a total of

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Data taken in part from a thesis to be submitted by the senior author to the Graduate School, University of Minnesota, in partial fulfillment of the requirements for the degree of master of science.

nine calcium experiments, three were fatal. These fatal experiments are indicated by dotted lines in figure 1. Table 1 gives average data for two of the fatal experiments (calves 877 and 950). Calf 951_2 was not included in table 1. In this animal, the dose, rate of injection, and blood level attained were considerably higher (7.81 mEq. of calcium/kg. in twenty minutes with the attainment of a serum-calcium level of 40.9 mEq./liter) than in the other 2 animals. This animal died immediately (fig. 1).

One animal (calf 877), given calcium chloride at a comparatively fast rate (0.18 mEq./kg./min. for twenty minutes), developed a total serum-calcium level of 14.3 mEq./liter, no higher than some of the other calcium-injected animals, yet this animal died. At the end of the injection, this calf appeared extremely weak and could not rise. Severe cyanosis, dyspnea, and heart block were present. In an effort to save the animal's life, 0.15 Gm. of potassium chloride were injected per kilogram of body weight. The calf appeared to respond well to this treatment and was considered to have made a satisfactory recovery. The animal was taken back to the stable but died approximately four hours later. Death would appear to have been due to the toxic effects of calcium, since its effects are known to be much more prolonged than those of postassium.[5, 6, 17, 21]

Calf 950 was given calcium chloride at a rate similar to that used in all other animals (fig. 1). This animal showed severe symptoms at a total serum-calcium level of 13.2 mEq./liter; when more calcium chloride was given, the animal died at the end of eighty-six minutes with a total serum-calcium level of 15.5 mEq./liter. A total dose of 6.04 mEq./kg. had been given. Thus, in this case, having a similar rate of injection to the others, the total dose administered and the blood level attained proved to be the main factors in causing death.

The maximum dose of calcium that seemed to be safely tolerated was approximately 0.07 mEq./kg./min. for forty-five minutes. When the rate or duration of injection exceeded these values, death occurred. The lethal total serum-calcium level was 14 to 16 mEq./liter. In one calcium gluconate experiment, the serum-calcium level reached 15.3 mEq./liter without causing death (fig. 1, and table 1). Higher blood levels were attained at lower doses for the calcium gluconate injections.

Death from calcium injections was due to cardiac failure, manifested by a severe degree of heart block and by ventricular fibrillation.[3] Severe cyanosis and dyspnea were also present. The animals appeared to become very weak and, later, prostrate. Depression and terminal excitability were noted. The animals which recovered

TABLE 1—Procedures Utilized in Intravenous Injection of Various Substances in Dairy Calves to the Point of Severe Symptoms or Death

No. of exp.	Substance injected	Blood level[a] in mEq./l., (ave.)	Termination of exp.	Cumulative dose/kg.		Duration	Rate
				Mg. of compound (ave.)	mEq. of ion (ave.)	Time in minutes (ave.)	mEq./kg./minute (ave.)
2[b]	10 % Ca gluconate	15.3	Recovery	746	3.33	61	0.06
4	6 % $CaCl_2$	12.1	Recovery	202	3.65	47	0.08
3[c]	6 % $CaCl_2$	14.9	Death	269	4.87	53	0.13
7[d]	3 % KCl	8.1	Recovery	306	4.11	99	0.04
1	8 % KCl	12.7	Death	629	8.43	168	0.05
4	12 % $MgCl_2$[e]	11.9	Recovery	613	6.05	74	0.08
1	12 % $MgCl_2$[e]	.....[f]	Death	244	2.40	17	0.14
1	$MgCl_2$[e]+	10.2 (Mg)	Recovery	473	4.66	53	0.09
	4.5 % $CaCl_2$	12.7 (Ca)		178	3.20		0.06
1	$MgCl_2$[e]+	15.6 (Mg)	Death	805	7.92	51	0.16
	3.5 % $CaCl_2$	19.0 (Ca)		235	4.22		0.08
2	1.5 % Na oxalate	2.4 (Ca)	Recovery	91	1.36	64	0.02

[a] All values are averages taken immediately after cessation of injections.

[b] Based upon only one experiment. In the other experiment, 0.74 mEq. of calcium were injected/kg. and only mild symptoms were observed.

[c] This table gives data for only two fatal calcium chloride experiments. In the third fatal experiment a dose of 433 mg./kg. was given in twenty minutes, resulting in a serum level of 40.9 mEq./liter.

[d] Based upon five experiments. The cumulative dose was unknown in two experiments.

[e] Magnesium chloride hexahydrate.

[f] Sample accidentally destroyed.

showed similar symptoms but to a lesser degree. Diuresis and lacrimation were noted toward the end of injections. The development of symptoms and cardiac disturbances seemed to be more immediate with calcium gluconate injections, but more severe and delayed effects were seen with calcium chloride injections. The time of death appeared difficult to predict, especially when calcium chloride was given. There did not seem to be a truly progressive development of symptoms, but rather unpredictable "attacks" occurred during which the symptoms appeared markedly aggravated. It was during these attacks that death occurred. It was felt that calf 877 died during one of these attacks since the total serum-calcium level at the time of death, four hours following cessation of injection, would not be expected to be high.

Potassium Administrations.—Of a total of eight potassium experiments, one was fatal (table 1 and fig. 2). It appeared that a lethal rate of injection *per se* was never attained in these experiments. Severe symptoms resulted with plasma-potassium levels of about 8 mEq./liter, and the single death was associated with the slow attainment of 12.7 mEq./liter over a period of 168 minutes.

In contrast to calcium injections, potassium injections were associated with gradual and progressive development of symptoms. The animals became extremely irritable, excitable, and could not stand. Diuresis became so marked that urination occurred every two or three minutes. The single death that occurred followed cardiac failure.[3] Cyanosis and dyspnea were not nearly as marked in these experiments as in the calcium experiments. After cessation of injections, recovery began immediately. The calcium experiments, as noted previously, did not show this progressive development and regression of symptoms.

Magnesium Administrations.—Of a total of five magnesium experiments, one was fatal (table 1 and fig. 3). Here again, the rate of injection seemed to be an important factor in causing death. In the single fatal experiment, the rate of injection was 0.14 mEq./kg./min., which rate was continued for seventeen minutes; in the four nonfatal experiments, the average rate was 0.08 mEq./kg./min., continued for an average period of seventy-four minutes. In the fatal experiment, death occurred even though the total dose was little more than one-third of the doses in the nonfatal experiments. Due to a laboratory accident, the plasma-magnesium levels could not be determined in this fatal experiment.

All of the magnesium injections were carried to the point of light anesthesia. This anesthesia occurred with the attainment of a plasma-magnesium level of 10 to 13 mEq./liter (fig. 3), and was preceded by a progressive dullness and depression. Extreme cyanosis and dyspnea were pres-

TABLE 2—Average Blood Electrolyte Levels in Dairy Calves Before and After Intravenous Injections with Various Substances

		Blood electrolyte levels in mEq./liter[a]											
		Total serum calcium		Plasma magnesium		Plasma potassium		Plasma[b] sodium		Plasma inorg. P		Blood glucose mg. per 100 ml.	
No. of exp.	Substance injected	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
8	Calcium[c]	5.1	12.8	1.9	2.0	4.8	4.5	145	139	5.1	5.0	76	93
8	Potassium chloride	5.2	4.9	1.9	1.9	4.5	8.6	145	143	4.6	3.8	84	68
4	Magnesium chloride	5.2	5.3	1.9	11.9	4.7	4.4	142	138	4.0	4.5	74	140
2	Magnesium chloride + Calcium chloride	5.4	15.9	2.2	12.9	4.3	4.0	.....	.....	3.3	3.4	84	141
2	Sodium oxalate	4.6	2.4	1.7	1.5	4.7	5.5	.....	.....	6.0	6.3	55	61
3	Glucose	5.1	4.3	1.9	1.6	4.8	4.3	.....	.....	6.1	5.4	80	660

[a] All values are averages taken before injections and immediately after cessation of injections.

[b] Sodium determinations were made on only four calcium, four potassium, and three magnesium experiments.

[c] Six of these experiments were injections of calcium chloride. Two were of calcium gluconate.

ent at this stage. The single death that occurred followed respiratory failure. After a given magnesium injection was stopped, progressive recovery seemed to take place

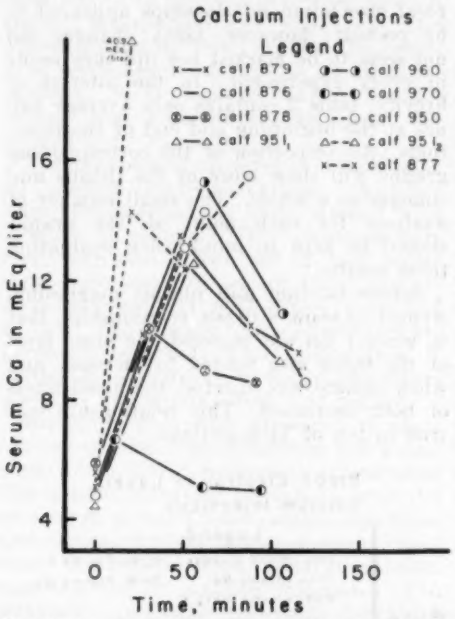

Fig. 1—Levels of total serum Ca attained with Ca injections. The half-solid circles (calves 960 and 970) represent Ca gluconate injections. All others represent $CaCl_2$ injections. Dotted lines indicate that these animals died.

in a manner similar to potassium injections.

Simultaneous Magnesium and Calcium Administrations.—Of the two experiments on the simultaneous injection of magnesium and calcium chloride, one was fatal (table 1). A lethal rate of injection of magnesium (0.16 mEq./kg./min.) was given in the fatal experiment but not a lethal rate of injection of calcium; however, this animal died, presumably of calcium toxicity at the end of the injection period of fifty-one minutes. The death appeared to be due to calcium because ventricular fibrillation occurred and respiratory movements continued for one to two minutes after the heart had stopped. The calf, however, had shown light anesthesia, presumably due to magnesium, for a period of six minutes before fibrillation began. Anesthesia did not occur in the nonfatal experiment, but the injection was stopped because of severe cardiac disturbance.

From an inspection of table 1, it appears that when magnesium and calcium were injected together, slightly higher blood levels of magnesium and calcium were necessary to produce anesthesia and ventricular fibrillation, respectively, than when magnesium and calcium were injected separately. However, higher blood levels of calcium were attained by lower total doses and rates of injection when calcium and magnesium were given together in these two experiments. Higher blood levels of magnesium did not seem to occur in this manner.

The symptoms exhibited by these 2 animals appeared to be the same as previously described for calcium and for magnesium, namely, cyanosis, dyspnea, depression followed by excitement, and in 1 case anesthesia and later death due to ventricular fibrillation.

Sodium Oxalate Administrations.—Sodium oxalate was injected in 2 different

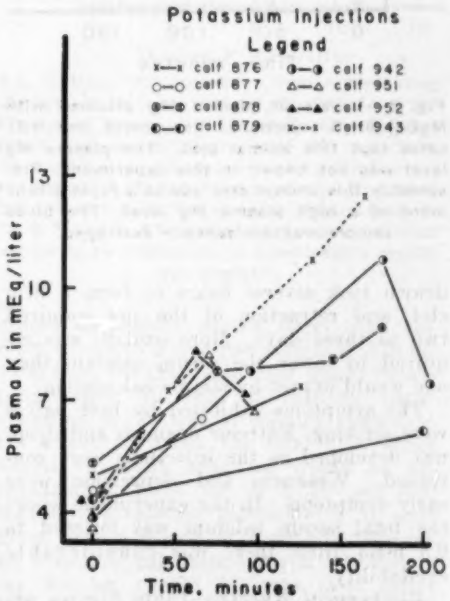

Fig. 2—Levels of plasma K attained with KCl injections. The dotted line indicates that this animal died.

calves without producing death (table 1). The total serum-calcium level was markedly depressed. In 1 calf, it was lowered to 0.8 mEq./liter. Blood samples that were drawn took several hours to form a firm clot, and retraction of the clot required two to three days. More oxalate was required to lower the serum calcium than one would expect by simple calculation.

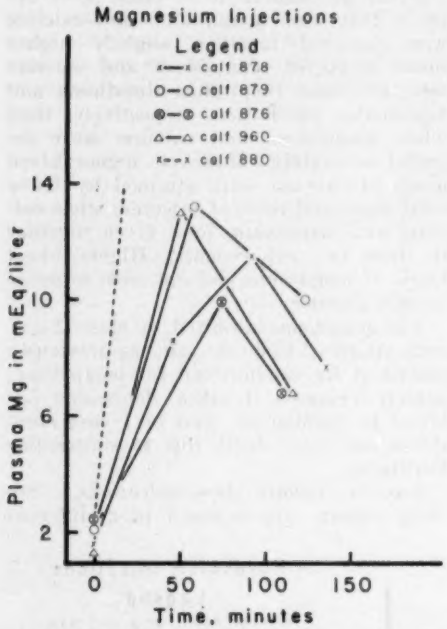

Fig. 3—Levels of plasma Mg attained with $MgCl_2 \cdot 6H_2O$ injections. The dotted line indicates that this animal died. The plasma Mg level was not known in this experiment. Presumably this animal died due to a rapid attainment of a high plasma Mg level. The blood sample was accidentally destroyed.

The symptoms exhibited by both calves were striking. Extreme cyanosis and dyspnea developed as the injections were continued. Weakness and depression were early symptoms. In the experiment where the total serum calcium was lowered to 0.8 mEq./liter, there was considerable excitability.

Biochemical Effects.—Table 2 gives average blood electrolyte levels before and immediately after cessation of injections for all types of experiments. Figures 4, 5, and 6 are graphic representations of these blood electrolyte levels for the injections of calcium, potassium, and magnesium, respectively. From table 2 and these three figures, it may be noted that several reciprocal electrolyte relationships appeared to be present; however, many changes did not seem to be marked nor did they occur in every experiment. In the interest of brevity, table 2 contains only average values at the beginning and end of the injections. An inspection of the corresponding graphs will show more of the details and changes as a whole. The small number of analyses for each point on the graphs should be kept in mind when evaluating these results.

Serum calcium and plasma magnesium seemed to show a direct relationship; that is, when 1 ion was injected, the blood level of the other also tended to increase, and when oxalate was injected, the blood levels of both decreased. This relationship was true in ten of 14 injections.

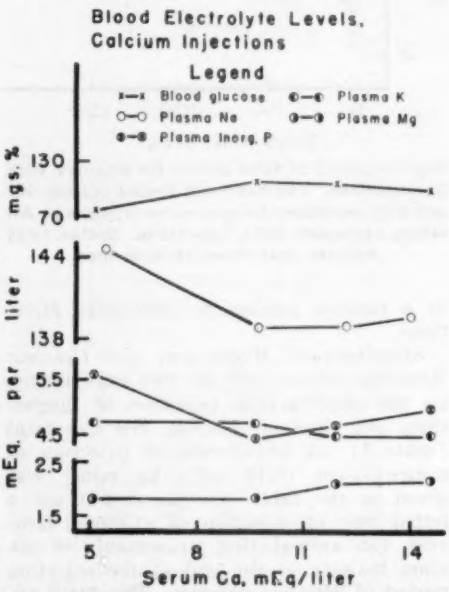

Fig. 4—Empirical regression lines representing changes in blood electrolyte levels with change in total serum Ca. All points are average taken from scatter diagrams of Ca injections. The number of blood analyses for each point for Na are 4, 3, 2, and 3, respectively. The number for each point for all other electrolytes are 10, 4, 5, and 5.

Potassium appeared to show an inverse relationship to both calcium and magnesium. When potassium was injected, the blood levels of both calcium and magnesium tended to decrease and when calcium and/or magnesium was injected, the plasma-potassium level usually decreased. This relationship was true in 13 of 16 injections. When sodium oxalate was injected, the plasma-potassium level increased quite markedly.

The plasma-sodium level decreased with all calcium and magnesium injections. This phenomenon was more marked with calcium injections. The plasma-sodium level decreased in two out of four potassium injections. In one of these, the sodium decreased 11 mEq./liter (from 146 to 135). The changes in the other three experiments were only 2 to 4 mEq./liter. A dotted line was drawn in figure 5 to represent this inadequate number of results.

Inorganic phosphate appeared to show inverse relationships to calcium and potassium but a direct relation to magnesium. The plasma-inorganic phosphate level decreased in seven of nine calcium injections and decreased in all eight potassium injections; it increased when the serum calcium was lowered by oxalate injections. The plasma-inorganic phosphate level increased slightly in all magnesium injections. It showed no marked change when magnesium and calcium were injected simultaneously.

Blood-glucose levels increased in all calcium and magnesium injections. This increase was especially marked with magnesium injections. With potassium injections, the glucose level decreased in all cases at the beginning, but in 2 animals it increased at the end of the injection. When the plasma-potassium levels became quite high, the animals showed marked excitability. Sodium oxalate injections were without regular effect on blood glucose. When large amounts of glucose were injected (average blood level of 360 mg. per 100 cc.), all blood electrolyte levels decreased.

Discussion

The maximum dose of calcium that seemed to be safely tolerated in these experiments was approximately 0.07 mEq/kg./min. for forty-five minutes. On the assumption that this dose would apply to adult cows, one can roughly calculate the total dose per adult animal. Calcium gluconate, however, would appear to be tolerated in slightly higher doses than calcium chloride. For a cow weighing 318 kg. (700 lb.), the total safe dose would be approximately 20 Gm. of calcium corresponding to 224 Gm. of calcium gluconate. On a solution basis, the total dose which would be safely tolerated would be approximately 1,120 ml. of a 20 per cent solution of calcium gluconate if it were given over a period of forty-five minutes. In a similar calculation for a cow weighing 455 kg. (1,000 lb.), the total safely tolerable dose would be 1,600 ml. of a 20 per cent solution if it were given over the same period of time.

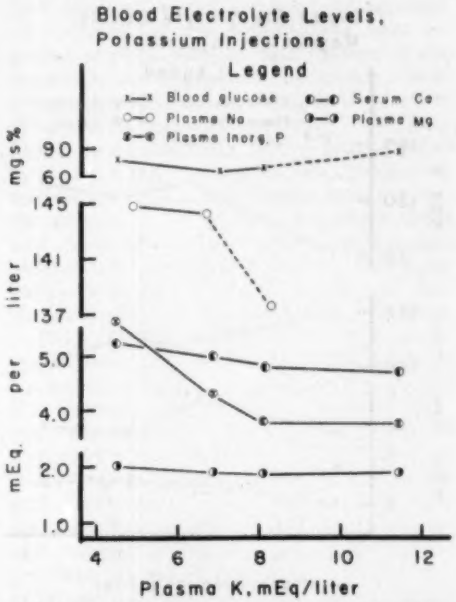

Fig. 5—Empirical regression lines representing changes in blood electrolyte levels with change in plasma K. All points are averages taken from scatter diagrams of KCl injections. The number of blood analyses for each point for Na are 4, 3, and 3 respectively. The number for each point for all other electrolytes are 12, 6, 7, and 3. The dotted lines represent what appear to be inadequate or questionable results (see context).

Since the present experiments were not designed to evaluate critically the safe rate of administration of a variety of commercially available preparations, only general

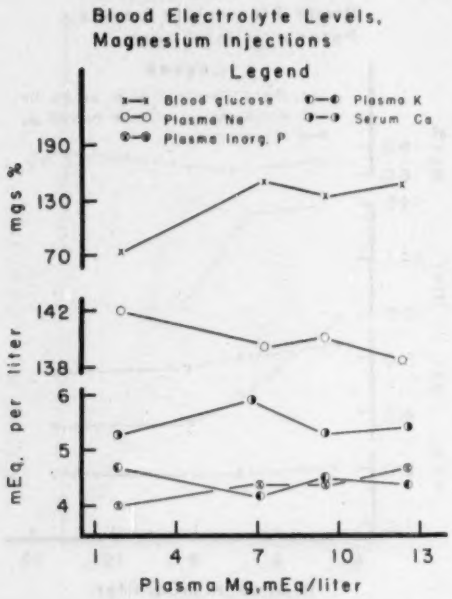

Fig. 6—Empirical regression lines representing changes in blood electrolyte levels with change in plasma Mg. All points are averages taken from scatter diagrams of Mg injections. The number of blood analyses for each point for Na and glucose are 3, 3, 3, and 2, respectively. The number for each point for all other electrolytes are 4, 2, 3, and 3.

indications are possible. Over the period of perhaps fifteen to twenty minutes, the average time usually employed for therapeutic injections of this sort, the total safely tolerable dose would be expected to be appreciably less than the amounts noted above.

Intravenous injections of calcium, potassium, and magnesium salts and sodium oxalate in high doses appear to initiate physiological disturbances in cattle, as in other species. The rate of injections, as well as the total dose and the blood level attained, seemed to be factors in causing death.

One might suppose that the faster the rate of administration, the less time the animal would have to adjust to, and compensate for, this sudden change in internal environment. As a result, these adjustments and compensations might be less efficient and the animal would manifest more severe symptoms with lower total doses and lower blood levels of the substance injected.

Fenn,[11] in his review, pointed out that when the serum-potassium level was very low, a sudden restoration of the serum potassium to normal values might elicit severe cardiac disturbances, presumably because of lack of time to recompensate to the normal serum-potassium level. Also, a rapid injection of potassium salts in a normal animal elicited an immediate ventricular fibrillation, whereas a slow injection led to a slow and progressive cardiac arrest.[11, 26]

Winkler, Hoff, and Smith[17, 26] stated that in dogs and human beings there were occasional sudden deaths when calcium was given intravenously in low doses. They attributed these sudden deaths to ventricular fibrillation. The initial stages of calcium toxicity were attributed to the vagal bradycardia but when rapid injections were made, only ventricular fibrillation was seen.

Delayed deaths in cattle and in dogs after calcium injections have been reported by Craige[5] and by Hoff *et al.*[17] respectively. These deaths have been attributed in general to circulatory embarrassment; the exact reasons for death at relatively low serum-calcium levels are obscure. In the present experiments, delayed symptoms occurred after calcium injections, and the animals were subject to periods of "attacks" during the injection and at variable times after cessation of the injections. It is known that calcium has some effects which are similar to those elicited by stimulation of the sympathetic nervous system.[20, 22] It might appear, therefore, that periods of excitement might increase the physiological effects of calcium due to the release of epinephrine, and thus help bring about these attacks. Therefore, when the animal had been under the burden of a rapid increase in the level of serum calcium at some time previously, a period of excitement due to any cause, such as noise, fright, etc., might suddenly be enough to potentiate the effects of calcium and cause death due to cardiac failure. It would seem that no epinephrine po-

tentiation would be likely in the case of potassium or magnesium since the effects of these ions have not been found to be sympathomimetic.

In the present study with magnesium injections, light anesthesia occurred with the attainment of a plasma-magnesium level of 10 to 13 mEq./liter. A progressive dullness, depression, and dyspnea was also associated with these experiments. A number of investigators [1, 10, 16, 18, 22, 25, 26] have reported on the narcotic, anesthetic, and lethal effects of magnesium in various animals. These authors noted that a depression occurred as the serum- or plasma-magnesium level rose above 4 mEq./liter, and complete anesthesia occurred when the serum-magnesium level reached 11 to 17 mEq./liter. These authors have also stated that calcium is an antagonist of magnesium narcosis and paralysis, and that when calcium is injected, the animals will suddenly awaken.

Smith,[25] however, has reported that in dogs injections of calcium hastened the occurrence of death in experimental hypermagnesemia and, instead, potassium appeared to be an antagonist of this magnesium toxicity. It was her opinion that when magnesium was injected, the anesthesia and paralysis were due to a direct effect of magnesium and also to an increased magnesium : potassium ratio; in other words, to an indirect potassium deficit. The serum-potassium level consistently fell, in her experiments; when this level was restored, the toxicity of magnesium was markedly reduced. As noted in figure 6, the plasma-potassium level in the present experiments tended to decrease when magnesium was injected into dairy calves.

Magnesium, besides having central nervous system depressant actions, also has effects similar to that of curare in that it blocks neuromuscular transmission.[10, 20, 22] In this way, it is said to produce a progressive paralysis, and death due to paralysis of the respiratory muscles. Potassium, on the other hand, has effects similar to that of acetyl choline in that it decurarizes or augments neuromuscular transmission.[7, 11, 20, 21] It has been stated by some of these authors that magnesium and potassium thus have somewhat opposite effects upon the nervous system. Calcium has effects somewhat similar to those of magnesium in that high serum-calcium levels have a depressant action upon the nervous system; yet when calcium and magnesium are given together, anesthesia is hindered or totally prevented. One would expect that potassium and magnesium, or potassium and calcium, would be good antagonists. The results of the experiments here reported tend to indicate that calcium is antagonistic to magnesium anesthesia; however, magnesium seems to have little effect in altering calcium toxicity. Slightly higher serum-calcium levels were needed to produce toxic effects in the presence of hypermagnesemia, but higher calcium levels seemed to be attained by lower doses and rates of injection when calcium and magnesium were given together. No explanation is at hand for this result, and further experiments would appear necessary.

The symptoms exhibited by the experimental lowering of the serum-calcium level by means of oxalate injection seem opposite to those of calcium injection in general. The effects of this artificially induced hypocalcemia were not as marked as those due to the injection of potassium. Dennis and Harbaugh [6] reported on the experimental alteration of the blood potassium and calcium levels in cattle and pointed out that the blood potassium : ionized calcium ratio seemed to be of great importance in the production of tetany. In our experiments, the plasma-potassium level increased when the serum calcium was lowered and this, therefore, would seem to contribute to the production of a higher potassium : calcium ratio and to the development of symptoms. Another factor that may possibly have led to the production of symptoms in our experiments was the simultaneous decrease in the plasma-magnesium level.

In the present study with dairy calves, the serum-calcium and plasma-magnesium levels seemed to show, on the average, a direct relationship to one another; the plasma potassium seemed to show an inverse relationship both to calcium and magnesium (fig. 4, 5, and 6 and table 2). Schmidt and Greenberg,[22] Fenn,[11] and Smith [25] also describe this same phenomenon in rabbits, dogs, and human beings. They state that when calcium was injected, the plasma-magnesium level increased but the reciprocal change, *i.e.*, increase in serum calcium when magnesium was injected, did not occur. Experiments cited

by these authors showed that the plasma-potassium levels decreased when calcium or magnesium was injected, and vice versa.

An interesting observation was that the average plasma-sodium levels decreased with calcium, potassium, and magnesium injections. The results of one experiment, however, considerably increased the average figure for the decrease in plasma sodium as observed in the potassium experiments (fig. 5). Sellers *et al.*[24] however, noted an increase in plasma and urinary sodium when potassium was given orally to adult cattle. The resultant plasma-potassium levels were not nearly as high or prolonged, however, as in the present experiments. It is known that potassium diffuses passively into cells and sodium is extruded from the cells,[7,11] although the extrusion of sodium is largely independent with respect to the time of entry of the potassium ion.[7] The increase in total urinary excretion of sodium during water and potassium diuresis[23,24] may serve as a possible explanation for the decrease of plasma sodium in the present experiments.

The decrease in plasma-inorganic phosphorus levels, when calcium was administered to cattle, is generally regarded to be a rather consistent response,[5,6,16,22] with the exception of the parturient period. It seems reasonable to suppose that the plasma-inorganic phosphorus level decreased as a result of calcium phosphate deposition in the bones. Another factor to consider is a probable increased urinary excretion or "washing out" of inorganic phosphorus following osmotic diuresis. An inspection of figure 4 reveals that the plasma-inorganic phosphorus level decreased to its lowest value at a total serum-calcium level of 10 mEq./liter but that it increased slightly as the serum-calcium level increased above this value. Craige *et al.*[6] has observed that high levels of serum calcium may actually reduce the volume of urine formed. It seems possible, then, that the excretion of inorganic phosphorus may thus be reduced by very high serum-calcium levels. A "shutting off" or depression of the parathyroid gland also may account for the slight increase in plasma-inorganic phosphorus levels.

A noticeable decrease of blood glucose and plasma inorganic phosphorus was present at sometime in all of the potassium experiments. In 2 of the 8 animals, late in the experiments, the blood-glucose level increased when the plasma-potassium level became markedly elevated. These animals showed marked excitability and it is supposed that this excitement and presumed release of epinephrine was a major factor in producing these high levels of blood glucose (dotted line of fig. 5). Sellers *et al.*[24] noted an increase in plasma inorganic phosphorus when potassium and sodium were given orally to adult cattle. Considerably higher plasma-potassium levels were attained in the present experiments, however. It is thought that inorganic phosphorus may be converted to organic phosphorus[11,22] in order to participate in the phosphorylation of glucose, and that when glucose is administered, the inorganic phosphorus level decreases. It is also believed[7,11] that potassium tends to be deposited and liberated with glycogen. It has been postulated[7,11] that potassium may have a cycle from muscle to liver, similar to the transference of glycogen from muscle to liver via blood glucose. This postulation is based upon the observation that blood potassium and glucose tend to rise and fall together, and upon the finding that when one of these two substances is injected, the blood level of the other will decrease because of increased tissue deposition of both substances. It appears, then, that potassium, inorganic phosphorus, and glucose are all related to the formation of glycogen, and that when one substance is injected, the blood levels of the other two substances may decrease. Another factor to be considered may be a "washing out" of inorganic phosphorus in the cases of both water and potassium diuresis.[23,24]

The blood-glucose levels increased in all of the calcium and markedly increased in all of the magnesium experiments. The handling of the animals during these experiments may very well have been contributory to this increase, but it was similar in all respects to the procedure used in potassium experiments, where a consistent fall in blood-glucose level was seen. Other investigators[1,10,22] have shown that magnesium injections are accompanied by marked increase in blood glucose but certain anesthetics are not, and that calcium injections are also accompanied by an increase in the blood-glucose level, and vice versa. No explanation is at hand for these responses.

SUMMARY

1) Dairy calves, weighing from 48 to 240 lb., were injected intravenously with calcium, potassium, and magnesium salts and sodium oxalate in an effort to learn more about their physiological and toxic effects.

2) A comparison of fatal and nonfatal experiments was made with regard to total dose, blood level attained, and rate of injection. When the rate of injection was high, smaller total doses and lower blood levels were needed to produce toxic effects.

3) Lethal blood levels attained were: 14 to 16 mEq. of total serum calcium per liter and 13 mEq. of plasma potassium per liter. Maximum safely tolerated rates of injection were: 0.07 mEq. of calcium/kg./min. for forty-five minutes, and 0.08 mEq. of magnesium/kg./min. for seventy-five minutes.

4) Delayed onset and development of symptoms with one death were noted with calcium injections, particularly in the case of calcium chloride injections. There did not seem to be a truly progressive development and regression of symptoms with calcium experiments, which is in contrast to the experience with the potassium, magnesium, and oxalate experiments.

5) When calcium and magnesium chloride were injected simultaneously, slightly higher blood levels of each were associated with the production of toxic effects. Calcium appeared to counteract somewhat the anesthetic abilities of magnesium. The reciprocal effect did not appear to occur.

6) Several electrolyte relationships were observed and discussed.

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Micrococcus Pyogenes in Bovine Milk
I. Identification

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IN THE development of bacterial flora in the bovine mammary gland, cocci are predominantly present and, under conditions favorable to their development, the pathogenic forms among them may produce inflammation of the gland, commonly called mastitis.

With the advancement of methods for identification and eradication of the streptococcic forms of this disease complex, staphylococcic mastitis has become of increasing importance.

The establishment of a criterion for pathogenicity among the members of the genus Staphylococcus, now termed Micrococcus, has proceeded slowly and numerous investigations have been concerned with studies on hemolysis, coagulation of plasma, pigment formation, liquefaction of gelatin, fermentation of carbohydrates, especially mannitol, and on other tests of particular value in the differentiation of pathogens from nonpathogens of this group. It is the aim of this paper to simplify, for practical routine use, the procedures requisite for detection and identification of pathogenic micrococci in bovine milk.

SOURCE OF MILK

Milk for these studies was obtained primarily from two self-contained herds, A and B. Herd A, consisting of about 110 cows in milk, has been under experimental investigation since 1938. The earlier years were devoted to studies on *Streptococcus agalactiae;* in 1947, upon eradication of this organism, methods for isolation of micrococci were initiated. Herd B, comprising about 225 cows in milk, has been under investigation since 1943; the earlier studies were likewise involved with eradication of *Str. agalactiae*, which was accomplished by 1945, and later work has been directed toward the micrococci. One other herd, C, was also under experimental study but most of the other milk samples examined were from clinical cases of mastitis or from animals representing herds in which clinical mastitis was occurring. These latter were selected for comparison with the herds under long-term study and were confined to a single observation.

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METHODS

Hotis.—About 18 ml. of milk were drawn aseptically into a vial containing 1 ml. of 0.33 per cent aqueous solution of bromcresol purple; the samples thus obtained were incubated about twenty hours and Hotis readings recorded. After streaking upon blood agar plates, they were reincubated another twenty hours and a forty-hour reading was then recorded.

Blood Agar Plate.—After the first incubation of the Hotis samples, a loopful from each was streaked upon one half of a plate of tryptose agar, pH 7.4, containing 5 per cent of washed bovine erythrocytes. Since it has not been feasible to use a calf (or sheep), as recommended by Mincett,[1] to avoid inhibition of toxin production by the natural antitoxins in older animals, mature bovine blood was used and the red cells washed three times in physiological saline to effect removal of antitoxins before the final suspension was made, approximating the original volume.

After twenty-four hours of incubation, the streaked plates were read and toxigenic colonies picked for the coagulase test.

Coagulase Test.—The coagulase test employed is a very slight modification of the method of Chapman, Berens, and Stiles.[2] Cultures were grown about eighteen hours on tryptose agar slants and 1 loopful of each culture was suspended in a tube containing 0.6 ml. of 0.4 per cent saline. Two-tenths milliliter of fresh rabbit plasma was added to each tube which was shaken and incubated in a water bath at 37 C. for four hours. The tubes were then removed and read; a second reading was recorded after the tests stood overnight at room temperature.

RESULTS

Toxin Production.—Cocci are tentatively identified as *Micrococcus pyogenes* when they exhibit in bovine blood agar reactions typical of the *alpha* and/or *beta* toxins. A clear hemolytic zone, with a radius usually of about 1 mm. immediately surrounding the colony, is called the *alpha* zone,[3] denoting the manifestation of *alpha* toxin.[4] A second wider zone of darkened red cells encircles the *alpha* zone and represents the elaboration of *beta* toxin.[3] Upon standing at room temperature, or in

the icebox, the *beta* zone clears, demonstrating "hot—cold" lysis. This darkened zone, after incubation at 37 C., has an average radius of about 4 mm. but may range from 2 to 10 mm. Colonies are recorded according to toxin type and radius of zone; for example, a colony with a *beta* zone of 4 mm. is expressed as B4, and a colony showing a 1 mm. *alpha* zone and a 4 mm. *beta* zone, as A1B4.

The great majority of toxigenic organisms observed in these studies manifested both *alpha* and *beta* toxins and in the special comparative tests to be reported in this paper the proportion of *alpha-beta* to *beta* only was approximately five to one. Not all colonies showing only the *beta* zones can be considered true *beta* types since, occasionally, colonies appearing to be *beta* types, upon restreaking, showed both zones. However, colonies showing either *alpha-beta* or *beta* toxin were picked routinely for conclusive differentiation by the coagulase test; colonies which showed only a clear hemolytic zone greater than 1 mm. in radius, or a *beta* zone so narrow as to be considered a questionable manifestation of toxin, were usually picked, also.

Coagulase Reaction.—Among some 2,200 coagulase tests on isolations, principally from herds A and B but including a very few isolations from nonmastitis cases or nonbovine sources, 2,084 were carried out on isolations manifesting *alpha-beta* or *beta* toxin, and 19 isolations, or less than 1 per cent, failed to produce coagulation of rabbit plasma. Of a total of 209 micrococci, either nonhemolytic or weakly hemolytic but showing no *beta* toxin, 17 were coagulase-positive and among them six weakly positive strains were in a group of 78 cultures recently isolated from herd B in which the percentage of infection with toxigenic micrococci has been markedly lowered.

A difference was observed in the coagulase reactions between the micrococcic isolations from herds A and B. Generally speaking, isolations from herd B showed a very strong positive reaction; that is, they produced a completely solid clot, or very nearly so, in contrast to isolations from herd A which coagulated the plasma much less solidly or even very weakly, with occasional partial or almost complete dissolution of the clot after overnight standing. Tests showing a solid clot almost invariably showed no appreciable dissolution of the clot upon standing.

The *beta* toxin zones observed in isolations from herd A have been predominantly narrower than those from herd B

TABLE 1—Comparison of Reactions Produced in Differential Mediums by Micrococcus Pyogenes Isolated from Milk Obtained from Cows Representing Various Dairy Herds

Herd	No. of isolations	Digestion of raw milk (Hotis test)	Litmus milk			PRM salt agar		Gelatin [1]
			ACR*	AC	A	Acid	alkaline	liquefied
A	91	7	6[2]	1	75	85[2]	1	2[2]
B	44	36	24	6	14	3[3]	32	4[3]
C	41	2	2	0	39[4]	41	0	3
D	73	8	0	68	5	72	1	3
E	26	26	25	1	0	26	0	22
F	20	6	2	10	8	20	0	0
G	5	5	0	0	5	....	....	....
H	1	0	0	0	1	1	0	0
I	11	9	0	1	10	11	0	0
J	6	....	2	0	4	6	0	0
K	3	1	0	0	3	3	0	0
L	13	0	0	12	1	13	0	0
M	7	0	0	5	2	7	0	0
N	3	1	0	2	1	3	0	2
O	5	....	0	3	2	5	0	0
P	3	0	0	1	2	3	0	0
Q	3	0	0	1	2	3	0	0
R	8	4	8	0	0	8	0	1

* ACR—Acid, coagulation, and reduction; AC—acid and coagulation; A—acid; PRM—phenol red mannitol.

[1]—Gelatin liquefaction determined on 72-hour growth at 37 C. transferred to refrigerator. Solidification in refrigerator recorded as negative result.

[2]—Total number of tests on L.M. = 82; on PRM salt agar and gelatin = 86.

[3]—Total number of tests on PRM salt agar = 35; on gelatin = 36.

[4]—Eleven also showed reduction.

and the possibility of a correlation between the reduced zone and weak coagulase production was considered but the few isolations from other herds which showed zones comparable in diameter to those from herd A did not appear to bear out this possibility. Most of the isolations from herds other than A have shown strongly positive coagulase reactions.

Comparative Tests.—Since it had been observed in herd B that Hotis milk samples from which *M. pyogenes* was isolated generally showed partial or complete digestion, frequently at the twenty-hour reading but definitely at the forty-hour reading, this finding was regarded as indicating the presence of the pathogen and was established in the Hotis code as the reading to be interpreted as characteristic for *M. pyogenes* in that herd.[5] Further observations in other herds showed that cocci toxigenic on blood plates and positive to the coagulase test did not uniformly digest the raw milk of the Hotis test. The possibility that other organisms present in the milk might prevent the characteristic digestion suggested the necessity of testing the reactions of single-colony isolations in raw milk containing bromeresol purple.

Field samples of milk giving Hotis negative readings after sixty hours of incubation were selected for inoculation with pure cultures of *M. pyogenes* to test for their ability to digest raw milk. In addition, reactions in litmus milk, Difco phenol red mannitol salt agar slants,[6] and Difco nutrient gelatin stabs were determined.

Each medium was inoculated with 0.1 ml. of broth culture and incubated for seventy-two hours. The Hotis test reactions were recorded at twenty and forty hours to make the readings conform to the practice employed with natural Hotis tests. The cultures in gelatin, together with a control, were iced after seventy-two hours of incubation, and called positive if they remained liquefied after the refrigeration. The results of the combined tests are given in table 1.

No significant correlation can be suggested between the results on raw milk, litmus milk, mannitol salt agar, and gelatin. Possibilities that hinted at some grouping of the isolations by the results of one or another of the tests were eliminated by the variabilities in the remaining tests. To illustrate, isolations from herds B and E showed similarity in their reactions on raw milk, and litmus milk but not in gelatin or in mannitol fermentation. In fact, the majority of isolations from herd B, in their alkaline reaction upon mannitol salt agar, differed almost completely from the isolations from all the other herds.

On the whole, the results of these four tests seem to indicate that they are not of particular value in differentiating pathogens from nonpathogens in the Micrococcus group but that they do reveal strain characteristics predominant in a herd.

DISCUSSION

The findings from these studies are in agreement with the work of Bryce and Rountree,[3] Minett,[1] and Slanetz[6] as to the critical importance of the production of *beta* toxin by *Micrococcus pyogenes* from animal sources. Inasmuch as the coagulase tests have exhibited such marked conformity with *beta* toxin production it would seem that, for practical application, manifestation of *beta* toxin should be sufficient evidence that the Micrococcus is *M. pyogenes*. In the rare instances in which colonies showing a single clearly hemolytic zone wider than 1 mm. in radius or when weakly or nonhemolytic micrococci only are observed in cases of clinical mastitis, the bovine blood plate method should be supplemented by coagulase test in order to prove pathogenicity.

The "characteristic" digestion of Hotis milk previously described as indicative of *M. pyogenes* should now be regarded as a strain reaction which may be predominant among the strains in certain herds but entirely absent among strains from other herds. Therefore, the reaction has become of diminished value in revealing the presence of pathogenic micrococci in milk by Hotis test alone.

CONCLUSIONS

The criteria for pathogenicity of the micrococci encountered in the udder are at present concluded to be: (1) production of *beta* toxin on agar containing blood capable of demonstrating it, and/or (2) the coagulation of rabbit plasma.

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Micrococcus Pyogenes in Bovine Milk
II. Relationship of Shedding Characteristics to Occurrence of Clinical Mastitis

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TWO LARGE dairy herds designated, respectively, as A and B in the previous paper [1] have been under long-term observation, first for investigation and eradication of *Streptococcus agalactiae* and, thereafter, for studies on *Micrococcus pyogenes*. Herd A was composed of about 110 cows in milk and herd B of about 225 in milk.

The laboratory procedures for isolation and identification of *M. pyogenes* have been described in part I of this series.[1] The technique employed for sampling of the milk, by the use of concentrated calcium hypochlorite and paper towels for teat disinfection, gave reasonable assurance that the bacteria isolated from the milk came from within the gland. For routine sampling, cow-composite milk samples were drawn but, in special instances, individual quarter samples were collected; the latter sampling was made usually at the beginning and end of a cow's lactation period.

Source of Micrococcus Pyogenes for Mammary Gland Infection.—Observations on herd B, already published,[2,3] are mentioned but briefly to serve as an indication of the source of *M. pyogenes*. Over a seven-year period of study, complete segregation of *M. pyogenes* shedders has been maintained and the conditions of management have been ideal for trials with various therapeutic agents for intramammary instillation on an extensive scale. In 1945, about 50 per cent of the cows were found to be shedding this organism and seven years of intensive control and therapy reduced the shedding to less than 5 per cent of the animals. Notwithstanding the ubiquity of *M. pyogenes* in nature, results in herd B suggest that the source of this pathogen for invasion of the mammary gland is primarily other infected or shedding glands. Spencer and Lasmanis,[4] attacking this problem differently, arrived at a similar conclusion.

Sensitivity of Micrococcus Pyogenes to Penicillin.—From March, 1949, to May, 1950, all isolations of *M. pyogenes* from both herds for coagulase tests were also tested for penicillin sensitivity. Tryptose agar plates were prepared, containing 0.01, 0.1, 1.0, and 10 units of penicillin*/ml. of agar. A loopful of eighteen-hour growth of each culture on agar slant was suspended in 1.0 ml. of normal saline, and a loopful of the suspension was then streaked on the surface of each of the test plates. The results of these and one later trial are presented in table 1.

TABLE 1—Penicillin Sensitivity of Isolations of Micrococcus Pyogenes from Herds A and B

Herd	No. *M. pyogenes* strains	Number and percentage of strains showing ability to grow on agar containing following penicillin unitage/ml.			
		0.01	0.10	1.0	10.0
A	370	366	270	209	67
		98.9	72.9	56.4	18.1
B	550	545	38	22	8
		99.1	6.9	4.0	1.5

Fifty-six per cent of the strains isolated from herd A grew in the presence of 1 unit of penicillin/ml. of agar and 18 per cent grew in the presence of 10 units/ml. However, only 4 per cent of isolations from herd B grew in the presence of 1 unit of penicillin/ml., and 1.5 per cent grew on agar containing 10 units/ml.

Occurrence of Micrococcus Pyogenes in Milk of Herd A.—There were no facilities for segregation in herd A nor was it possible to employ therapy on a large scale. Some attempt was made to milk the heifers first, but outside the milk barn they had direct contact with the older cows. Therapy, in general, was limited in use to individual glands showing visible symptoms of mastitis. The milkers reported such occurrences immediately upon detection and intramammary therapy was initiated by the herdsman. Penicillin in 250,000- or 500,000-unit doses was infused and repeated at 24-hour intervals until four infusions were given. The majority of cases responded satisfactorily, although complete sterilization of the gland seldom resulted. Instances not responding to this treatment, or of long-standing infections with previous clinical flare-ups, were given the larger dose of penicillin in combination with 1 Gm. of dihydrostreptomycin at each infusion.

The limited segregation and the restricted use of antibiotics in the presence of a high degree of resistance to the penicillin probably did not interfere greatly with the spread of *M. pyogenes*. The data on occurrence of this pathogen in the milk as found in periodic herd surveys made over a six-year period, extending from January, 1947, to January, 1953, may serve as an example of the

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* Standardized penicillin, 1,000 units/ml. of solution was supplied by Cutter Laboratories, Berkeley, Calif.

patterns of *M. pyogenes* shedding when natural spread is uncontrolled in a large milking herd.

Between November, 1948, and August, 1949, weekly visits were usually made and, during that period, selected cows, mainly first lactation animals, were sampled at each visit on an individual quarter sample basis. At the same time, a portion of the remainder of the herd was included for composite sampling on each trip. Of the seventy-two months in the six-year period of observations, herd analysis has necessarily been limited to forty-eight months because of lapses between tests. The greatest lapses between consecutive tests were one period of five months, one of three, and one of two months. Incidence of shedding *M. pyogenes* varied from a low of 25.6 per cent of the herd in July, 1949, to a high of 75 per cent in June, 1950. Average incidence by years from 1947 through 1952 was, respectively, 43, 49, 47, 64, 57, and 62 per cent.

The marked increase in isolations of *M. pyogenes* in the last three years is, in part, due to improvement in the sampling technique and changes in laboratory methods, such as the substitution of tryptose agar for veal infusion agar used previously, and the more recent practice of inoculating phenol red mannitol salt agar[6] with incubated milk when the streaked blood agar plates were over grown by coliform organisms or certain other bacilli.

Comparison of results with milk from individual cows disclosed that some cows shed the pathogen not at all, or infrequently; others shed it intermittently at short intervals and still others showed a persistent shedder status, extending over several lactation periods. The data on 4 representative animals are shown in table 2. Cow 354 shed *M. pyogenes* during her first lactation from one or more quarters in 25 of 29 tests made at short intervals, and the pathogen was found on each composite test on her milk collected in the second through the fifth lactation. In contrast, cow 350 was sampled 31 times on a quarter sample basis during her first lactation and at no time was *M. pyogenes* observed. The milk of this cow was essentially free of the pathogen during her second and third lactations also; whereas in the fourth lactation, it was present in 80 per cent of composite milk samples. The 2 remaining cows depicted in the table were intermediate between these extremes of shedding *M. pyogenes*.

The frequency of occurrence of *M. pyogenes* in the milk collected over the six-year period is correlated with lactation age in table 3. The cows within each lactation age are arranged in four groups; namely, nonshedders, shedders of *M. pyogenes* in less than 25 per cent of their milk samples, in 25 to 50 per cent, and in more than 50 per cent of the samples collected within the lactation period. The number of cows included was 207 and total number of lactations was 657.

Shedding of *M. pyogenes* at the highest rate was observed to increase with advancing number of lactations until a maximum of about 70 per cent of the cows was reached in the fifth lactation. In the sixth lactation, less than 2 per cent of the cows were entirely free of *M. pyogenes* in their milk; whereas the percentage shedding at a rate higher than 50 per cent decreased over that observed in the fifth lactation, and the number shedding at the 25 to 50 per cent level increased. In the seventh and subsequent lactations, the percentage that showed no shedding increased and varied between 7.0 and 14.0. While the group

TABLE 2—Examples of Occurrence of Micrococcus Pyogenes in Individual Quarter Samples of Milk in First Lactation Animals Compared with Incidence in Composite Samples Collected in Succeeding Lactations

Cow*		Nov.**	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Percentage incidence of *M. pyogenes* in composite samples drawn in subsequent lactations. 2nd	3rd	4th	5th	6th
	A	o o o	s o o	s o s s	o o o o	o o	o o o	o o o	o o o	o o	o o					
354	B	o s s	s s s	s s s s	o o o o	o o	s o o	s s s	o o o	s o	o o	100	100	100	100	
	C	o o o	s s s	s s s s	s o o o	o s	o o s	o o o	o o o	o o	o o					
	D	o o o	s s s	s s s s	s s s s	s s	s s s	s s s	o s o	s o	s s					
	A	o o o o	o o o	o o o o	o o o o	o o o	o o o	o o o	o o o	o o	o o					
350	B	o o o o	o o o	o o o o	o o o o	o o o	o o o	o o o	o o o	o o	o o	10	0	80	.....	
	C	o o o o	o o o	o o o o	o o o o	o o o	o o o	o o o	o o o	o o	o o					
	D	o o o o	o o o	o o o o	o o o o	o o o	o o o	o o o	o o o	o o	o o					
	A	o o o o	o o o	o o o o	o o o o	o o o	o o o	s o o	o o o	o o	o o					
600	B	o o o o	o o o	o o o o	o o o o	o o o	o o o	o o o	o o o	o o	o o	100	100	100	100	
	C	o o o o	o o o	o o o o	o o o o	o o o	o o o	o o o	o o o	o o	o o					
	D	o o o o	o o o	o o o o	o o o o	o o o	o o o	o o o	s o o	o o	o o					
	A	Freshened		o o o o	o o o o	s o s	s s s	s s o	o o o	o o	o o					
615	B	in Jan.		o o o o	o o o o	o o o	o o o	o o o	o o o	o o	o o	44	60	66	.....	
	C			o o o o	o o o o	o o o	o o o	s o o	o o o	o o	o o					
	D			o o o o	o o s s	o o o	o o o	s o o	o o o	o o	o o					

* A—right front; B—right rear; C—left front; and D—left rear; ** o—*M. pyogenes* not present; s—*M. pyogenes* present.

shedding *M. pyogenes* in their milk in more than 50 per cent of samples increased with lactation age to a maximum at about the fifth lactation, some cows that had shed at that high level in one lactation changed to a lower rate of shedding in the next lactation. The shedding groups into which high-shedder cows fell in the second of each paired lactations are shown in table 4. Here it is seen that among cows that had had *M. pyogenes* in their milk at the highest level, between 9 and 28 per cent shed the pathogen at a lesser rate in the lactation immediately following. The least change occurred between the second and third lactations and the greatest change in shedding rate appeared between the fifth and sixth lactations.

Occurrence of Clinical Mastitis in Herd A.—Since it was not possible to study the milks for evidence of subclinical mastitis, only the instances in which visible symptoms appeared, such as swelling of the gland or discernible alterations occurring in the milk, have been included in this study. A total of 75 lactations was recorded as showing clinical mastitis and this is correlated in table 5 with the frequency of shedding *M. pyogenes*. Here it can be seen that 85 per cent of the total occurrence of clinical mastitis appeared in the lactations showing the highest shedding rate, 13 per cent in the lactations shedding at the 25 to 50 per cent level, and less than 2 per cent of the cases fell into the lowest shedder group. It is also shown that of a total of 341 lactations associated with *M. pyogenes* in more than 50 per cent of the randomly collected milk samples, the occurrence of clinical mastitis was limited to 18.7 per cent of these lactations.

TABLE 3—Frequency of Occurrence of Micrococcus Pyogenes in Milk Samples Collected from Herd A Over a Six-Year Period, Correlated with Lactation Age

Lactation age or calvings	No.* of lactations	Percentage of lactations in each of the four frequencies of shedding *M. pyogenes*.			
		0 %	Less than 25 %	25 % - 50 %	Over 50 %
1	114	36.0	20.0	23.7	20.0
2	103	30.0	5.8	28.2	36.0
3	80	20.0	3.7	21.2	55.0
4	78	11.5	12.8	16.7	59.0
5	74	9.4	9.4	10.8	70.0
6	66	1.5	10.6	28.8	59.0
7	50	10.0	12.0	8.0	70.0
8	42	7.1	7.1	11.9	74.0
9-13	50	14.0	6.0	12.0	68.0

* 207 individual cows with total of 657 lactations.

DISCUSSION

The occurrence of such high percentages of shedders of *M. pyogenes* in herds A and B is not a unique observation. Similar findings have been made in many additional herds, both small and large. Others have also shown *M. pyogenes* to be a significant organism in udder flora. Minett [6] reported it in 34.9 per cent of 415 cows in five herds. The largest herd contained 186 cows and the incidence was 62 per cent. Gwatkin and Hadwen [7] state that in a series of 260 cows, 143 were affected with mastitis of which 30 cases were due to *M. pyogenes*. Of interest, in the latters' work, is the fact that 14 of the cases of clinical mastitis occurred in a single 18-cow dairy.

Investigators [6-10] agree that micrococcic mastitis is, in general, mild in character, but that severe cases may occur. The tissue reactions usually remain subclinical in that abnormalities of the milk are limited to increases in chloride content and leukocyte number. In addition, shedder cows

TABLE 4—Shedding Rates of Micrococcus Pyogenes in the Second of Paired Lactations among Cows Shedding at More than a 50 per cent Rate in the First of the Paired Lactations

Lactation pair		1 and 2	2 and 3	3 and 4	4 and 5	5 and 6	6 and 7	7 and 8	8 and 9
No. of Cows		21	23	30	28	36	24	25	18
Cows in each shedding group in the second of the paired lactations (%)	* 0	0.0	0.0	3.0	4.0	0.0	0.0	0.0	0.0
	1	5.0	0.0	7.0	4.0	3.0	8.3	8.0	11.0
	2	9.0	9.0	7.0	14.0	25.0	8.3	4.0	11.0
	3	86.0	91.0	83.0	78.0	72.0	83.3	88.0	78.0

* 0—*M. pyogenes* negative; 1—*M. pyogenes* in less than 25 % of the samples; 2—*M. pyogenes* in 25 to 50 % of the samples; 3—*M. pyogenes* in more than 50 % of the samples.

may show temporary or intermittent swelling of the gland and visible alteration of the milk, or both, and finally sporadic cases of fulminating gangrenous or non-gangrenous mastitis occur in some, but not all, infected herds. The reasons for such variations, in response to the presence of *M. pyogenes* in the mammary gland, are little understood. Since this pathogen is a tissue invader and produces a dermonecrotic toxin, the *alpha* toxin, it is conceivable that the intensity of tissue response is, in part at least, a function of tissue penetration and toxin production.

Frequency of the occurrence of *M. pyogenes* in the milk was found, in general, to be directly related to increasing lactation age. The fact that 20 per cent of heifers (table 3) showed a high rate of shedding during their first lactation, and continued to shed at the same high level in immediately succeeding lactations, suggests that this relationship is not entirely the natural consequence of repeated exposure to this organism but may also be related to a receptiveness on the part of the mammary tissue which may be a prerequisite to establishment of the pathogen within the gland. With the exception of the above mentioned heifers which did not fit the usual pattern, 30 per cent of the heifers remained free of infection and 50 per cent shed intermittently during their first lactation. In each succeeding lactation, fewer cows remained free and an ever greater number advanced to an intermittent or persistent shedder status so that, by the time the fifth lactation was reached, 70 per cent were shedding at the highest rate (table 4).

It has previously been hypothecated that the pathogenesis of *M. pyogenes* in the mammary gland proceeds from a free-living stage in the milk channels through two stages of tissue invasion. These new observations on frequency of shedding of this organism in relation to occurrence of clinical mastitis appear to fit the hypothesis; for example, the occurrence of the pathogen in less than 25 per cent of the samples could be correlated with less than 2 per cent of the observed cases of clinical mastitis. Occurrence in 25 to 50 per cent of the samples was correlated with 13 per cent of the observed mastitis and shedding of the organism in more than 50 per cent of the samples, with 85 per cent of the clinical cases (table 5). The lowest level of shedding may be representative of the free-living stage in the milk channels; the intermittent level of shedding, with both the free-living stage and second stage, or initial penetration of tissue, and the highest level of shedding may be representative of the third stage, or frank tissue invasion.

TABLE 5—Correlation of Clinical Mastitis with Rate of Shedding of Micrococcus Pyogenes

Frequency of shedding	None	In less than 25 % of milks	In 25-50 % of milks	In greater than 50 % of milks
Number of lactations	120	68	128	341
Lactations in which clinical mastitis was observed (%)	0	1.4	7.8	18.7
Distribution of total occurrence of clinical mastitis* (%)	0	1.3	13.3	85.3

* Clinical mastitis was limited to 75 lactations among a total of 657 lactations.

Although a very great percentage of the observed clinical mastitis occurred among those lactations associated with the shedding of *M. pyogenes* in more than one half of the milks tested, clinical symptoms appeared in but 18.7 per cent of a total of 341 lactations falling in that shedder-group. This is regarded as a low incidence of clinical mastitis in the presence of a high level of shedding and it may reflect the development of a resistance to the pathogen.

A further possible indication of developing resistance is the finding that some cows after having shown a high level of shedding subsequently enter an entirely negative phase or a period of shedding at a reduced rate (table 4).

The low incidence and general mildness of mastitis associated with *M. pyogenes* may be the result of a development of antitoxin titers. That micrococcic antitoxin titers do occur in bovine blood, colostrum, and milk has been shown by Minett[11] and Miller and Heishman;[12] the former investigator has stated that high antitoxin titers in bovine blood can be correlated with advancing age of the animals. The disappearance of the organism from the milk of cows previously observed to be shedding at a high rate might possibly

be related also to the action of bacteriophage.[13, 14]

CONCLUSIONS

1) The source of *Micrococcus pyogenes* for infection of the bovine mammary gland is essentially other infected or shedding glands.

2) *Micrococcus pyogenes* isolated from milk may show considerable resistance to penicillin and the percentage of resistance strains may vary among herds.

3) Consecutive complete herd surveys for *M. pyogenes* in the milk may reveal considerable variation from test to test. The average incidence is high and may exceed 50 per cent of the cows.

4) In general, a direct correlation exists between advancement in lactation age and a high level of shedding of *M. pyogenes*.

5) Clinical mastitis due to *M. pyogenes* was related to the rate or persistency of shedding the pathogen in the milk.

6) Some presented evidence indicates the probable development of resistance to *M. pyogenes* among infected cows.

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On the Essentiality of the Bovine Corpus Luteum of Pregnancy

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THE NECESSITY of the corpus luteum to maintain the uterus in a state compatible with pregnancy in the cow has been a matter of dispute.

Hess[1] and Schmaltz[2] reported that removal of the corpus luteum during early pregnancy terminated pregnancy in the cow. Sartoris[3] removed either both ovaries or the ovary containing the corpus luteum from 6 cows pregnant more than six months and observed abortions in all cases. Wester[4] stated that removal of a cow's ovary containing the corpus luteum resulted in death of the fetus.

Marshall and Jolly[5] and Kleinhaus and Schenk[6] reported that the corpus luteum was not necessary for fetal life and the continuance of pregnancy during the latter half of the gestation period in the cow. More recently, Uren and Raeside[7] showed that removal of the corpus luteum from a heifer on the 205th day of pregnancy did not cause abortion while removal of the corpus luteum of a heifer on the 177th day of pregnancy was followed by abortion.

Pregnancy in the rat,[8] goat,[9] and rabbit[10] is dependent on an intact corpus luteum throughout most, if not all, of gestation, whereas pregnancy in the sheep,[11] monkey,[12] guinea pig,[13] dog and cat,[14] woman,[15] and mare[16] is not dependent on an intact corpus luteum throughout gestation.

This study was initiated to seek information on the essentiality of the corpus luteum during various stages of pregnancy of the cow. This information is pertinent in order to evaluate the possible role of the corpus luteum in habitual abortion in cattle, the feasibility of corpus luteum expression to terminate late pregnancy in the cow, and as a background for other studies of corpus luteum dysfunction.

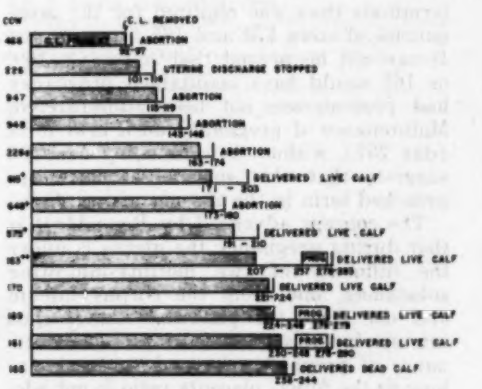
MATERIALS AND METHODS

One Guernsey (cow 815) and 12 Holstein-Friesian cows were used in this study. These cows were negative to blood serum-agglutination tests for brucellosis before and during the experiment. Breeding was by natural service and day one of pregnancy was considered the day following insemination. A careful record of breeding dates and estrous periods were kept on all cows, except 3 animals purchased while pregnant, and 1 cow whose breeding date was not recorded. Pregnancy and breeding date (except 3 purchased cows) were confirmed by rectal palpation. The breeding dates of the 3 purchased cows were estimated from crown-rump measurements of the aborted fetuses.[17] The breeding date of the cow not recorded was estimated from a rectal examination on approximately the fiftieth day of pregnancy.

The corpus luteum was manually enucleated from the ovary and removed from the body cavity in all cases. The peritoneal cavity was entered through vaginal incisions in 10 cows and through incisions in the abdominal wall of 3 cows. Daily observations were made for abortions or discharges of a macerated fetus. Periodic rectal examinations of each fetus were made during the experiment.

RESULTS

Removal of the corpus luteum between the ninety-second and 191st days of gestation was followed by the termination of pregnancy (chart 1). Four to thirty-two days elapsed between corpus removal and expulsion of the fetus. The fetus was expelled intact in all except 1 case, cow 226,



+ Breeding date determined from crown-rump measurements of fetus at delivery.

++ Breeding date estimated from rectal palpation on approximately fiftieth day of gestation.

Chart 1—Relationship of the corpus luteum to the termination of pregnancy.

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which discharged a macerated fetus until thirteen days after corpus luteum removal.

The results were less consistent when the corpora were removed between the 207th and 236th days of pregnancy. Cow 170 delivered a live calf three days after corpus removal on the 221st day of the gestation period, and cow 165 delivered a dead calf eight days after removal of the corpus on day 236 of the gestation period. Three cows, 163, 169, and 161, were subjected to removal of corpora on days 207, 224, and 230 of the gestation period, respectively, and maintained pregnancy for fifty, twenty-four, and eighteen days, respectively, before daily injections of 100 mg. of progesterone in sesame oil were begun. The progesterone injections were a part of another study, but these data are presented here for consideration. These 3 cows delivered viable calves five, one, and two days, respectively, after discontinuance of injections on day 278 of pregnancy. All calves delivered after day 202 of gestation were alive at birth except that from cow 165 (chart 1). The calves from cows 815, 575, and 170 died within three hours after delivery.

DISCUSSION

These data (chart 1) indicate that the corpus luteum is necessary for the maintenance of pregnancy in some cows as late as day 236. The pregnancies of cows 163, 169, and 161 had a longer period in which to terminate than was required for the pregnancies of cows 170 and 165 to terminate. It can not be proved that cows 163, 169, or 161 would have maintained pregnancy had progesterone not been administered. Maintenance of pregnancy until near term (day 257), without injections, by cow 163 suggests that this cow might have approached term in the absence of injections.

The concept advanced by Reynolds [18] is that during pregnancy the uterus is under the influence of two motility-inhibiting substances, one from the corpus luteum and one from the placenta. The corpus luteum remains essential to the maintenance of pregnancy in a given species so long as the fetus : placenta ratio is not adequate to maintain the pregnancy through the means of the placenta alone. In the rabbit this state is never reached, while in the monkey it is reached fairly early in the course of pregnancy. From our data, in the cow, it appears to be reached quite late in pregnancy, if at all. Quite likely an individual variation exists after approximately the 200th day of pregnancy.

Previously published work from this laboratory [19] demonstrated the need for progesterone injections only until day 162 of the gestation period in order to maintain pregnancy when the corpus was removed at day 60 of pregnancy. This dispensability of progesterone (from the corpus or replacement therapy) is markedly earlier (by approximately thirty days) than the data indicate in this paper, whereby removal of the corpus as late as day 191 was followed by premature delivery. Several explanations can be offered for this difference although no supporting data can be presented. The cows which aborted when the corpus luteum was removed between the 160th and 190th day of pregnancy were not maintaining pregnancy under an abnormal condition. The cows which were receiving injections (in the absence of a corpus) maintained pregnancy, yet that is our only proof of complete replacement therapy. Perhaps there was a chronic progesterone deficiency in those animals which received injections that stimulated compensation by another organ. There is no direct evidence that any other organ elaborates progesterone but Henry *et al.*[20] found pregnandiol in the urine of pregnant castrated women during late pregnancy. Hypotheses have been advanced that the placenta [21] or adrenal cortex [18] may elaborate progesterone during late pregnancy. Desoxycorticosterone as well as progesterone will maintain pregnancy in the cat [22] and the rabbit.[23] It seems possible that the cows which maintained early pregnancy without intact corpora (but received progesterone injections) may have been dependent upon another organ for the elaboration of progesterone or a like substance to a greater extent than those animals which had intact corpora lutea. Then, when the injections were discontinued in the one group and the corpora lutea removed in the other, the former group could rely to a greater extent on another endogenous source of progesterone, or a progesterone-like substance.

The effects of surgery cannot be discounted in the latter group when it is con-

sidered that the surgery came at the same time as the loss of endogenous progesterone which was not replaced.

SUMMARY

The necessity of the corpus luteum for the maintenance of pregnancy in the cow was studied by surgical ablation at different stages of pregnancy.

Pregnancy in 10 cows terminated following surgical removal of the corpus luteum between days ninety-two and 236 of pregnancy.

Corpus luteum removal from three cows on days 207, 224, and 230 was followed by pregnancy maintenance fifty, twenty-four, and eighteen days thereafter, respectively, when transferred to another experiment.

It appears that the corpus luteum is necessary to maintain pregnancy until approximately the 200th day of pregnancy, thereafter an individual variation exists.

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Qualitative Investigation of Estrogens and Testosterone of Bull Semen

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CERTAIN physiological processes of the semen during reproduction are not well understood as yet despite the fact that important knowledge about its composition and function have been learned recently. Inasmuch as there are no data available in the literature concerning hormonal composition of the semen, it was thought necessary to investigate this matter to facilitate a better understanding of the role played by the semen during and after its ejaculation in the vagina of the female.

Bull semen was chosen for this research. The study was conducted to determine whether or not estrogens and testosterone are present in bull semen. Ether extracts of bull semen and also pure bull semen were used for the biological assays.

MATERIALS AND METHODS

Two different kinds of bull semen were used in these experiments. Both were from Holstein-Friesian bulls used for artificial insemination purposes; they were of proved high fertility rate. At time of collection, both samples of semen showed about 75 per cent motility, good morphology, and high spermatozoid count. Collections were made by artificial vagina and kept in sterile glassware at room temperature for a few minutes before use.

Ether extracts from 10 cc. of each kind of bull semen were prepared in accordance with the method of Friedgood *et al.*[1] with the idea of obtaining estrogenic and testosterone fractions. These fractions were assayed for estrogens and testosterone, respectively, using female and male albino rats.

Estrogen Assay.—Two different methods of assays were used: (1) Astwood's[2] assay consisting of the increase of uterus weight of immature female albino rats six hours after subcutaneous injection of estrogens. Twelve immature (21 days old) female albino rats were used; their body weight averaged between 54.9 and 61.2 Gm. Of these, 6 rats were treated with a subcutaneous injection of a mixture of 2 cc. of the ether extract of the estrogenic fraction of bull semen and 2 cc. of peanut oil. The other group of 6 rats were used as controls and received a subcutaneous injection of 2 cc. of peanut oil.

Another group of 12 immature (21 days old) female albino rats averaging between 54.5 and 62.0 Gm. were also assayed for estrogens using the same method as described above but this time using subcutaneous injections of pure bull semen. A group of 6 rats received 2 cc. of pure bull semen and 6 served as controls, receiving 2 cc. of physiological saline solution. Six hours after the injections, all the rats were killed, their uteri carefully dissected and weighed on a precision balance.

2) Allen and Doisy[3] assay consisting of the cornification of the vaginal epithelium of spayed female mature albino rats twenty-four or thirty-six hours after the deposition of estrogens in the rat's vagina.

Eight female mature albino rats were spayed and used for the assay ten days after spaying. Of these, 5 received a drop of the ether extract of the estrogenic fraction of bull semen in the vagina, and 3 served as controls. Another group of 8 female spayed, mature albino rats were assayed with pure bull semen. Of these, 5 received a drop of pure bull semen in the vagina, and 3 served as controls.

Vaginal smears from all rats were microscopically examined after twenty-four and thirty-six hours.

Assay of Testosterone.—The Moore *et al.*[4,5] assays, consisting of the increase in weight and also the epithelial response of the seminal vesicle of mature, male castrated rats after the intraseminal vesicle injection of testosterone, were used.

Sixteen mature, male albino rats averaging between 317.0 and 338.1 Gm. of body weight were castrated. Fourteen days after castration 4 rats received an intraseminal vesicle injection in their right seminal vesicle consisting of a mixture of 0.05 cc. of the ether extract of the testosterone fraction of bull semen and 0.05 cc. of peanut oil. Their left seminal vesicles were injected with 0.10 cc. of peanut oil, serving as local controls.

Another group of 4 rats received an intraseminal vesicle injection of 0.1 cc. of pure bull semen in their right seminal vesicle and 0.1 cc. of physiological saline solution in their left, serving thus as local controls.

Finally, the remaining 8 castrated rats received no treatment and were considered as controls, 4 rats for each experiment.

This work was conducted at Cornell University. At present, Dr. Puntriano is at the Wyoming State Veterinary Laboratory, Laramie.

The author is indebted to Drs. S. A. Asdell, W. Hansel, and S. L. Leonard of Cornell University for the laboratory facilities extended during the conduction of the experiment, and to Dr. R. W. Bratton for supplying the samples of bull semen from the Artificial Insemination Laboratory of Cornell University.

TABLE 1—Results of Estrogen Assay of the Ether Extract of the Estrogenic Fraction of Bull Semen Using Astwood[2] Assay Method

Controls			Experiment		
Rats inj. subc. with peanut oil			Rats inj. subc. with ether extract of the estrogenic fraction-peanut oil mixture		
Rat No.	Body wt. (Gm.)	Uterus wt. (mg.) 6 hr. after inj.	Rat No.	Body wt. (Gm.)	Uterus wt. (mg.) 6 hr. after inj.
1	58.0	25.6	7	55.5	25.4
2	55.8	28.0	8	56.0	26.2
3	55.7	25.9	9	58.3	30.1
4	60.0	27.7	10	55.3	27.3
5	61.2	30.1	11	54.9	24.2
6	58.0	26.4	12	60.2	27.0
Average		27.2	Average		26.7

TABLE 2—Results of Estrogen Assay of Pure Bull Semen Using Astwood[2] Assay Method

Controls			Experiment		
Rats inj. subc. with peanut oil			Rats inj. subc. with pure bull semen		
Rat No.	Body wt. (Gm.)	Uterus wt. (mg.) 6 hr. after inj.	Rat No.	Body wt. (Gm.)	Uterus wt. (mg.) 6 hr. after inj.
1	62.0	32.8	7	56.3	24.5
2	54.5	24.0	8	Died	.....
3	57.0	28.9	9	59.2	27.0
4	57.5	26.2	10	60.3	28.5
5	60.0	29.1	11	54.9	26.8
6	58.3	27.0	12	58.7	27.3
Average		28.0	Average		26.8

TABLE 3—Results of Estrogen Assay of the Ether Extract of the Estrogenic Fraction of Bull Semen Using Allen and Doisy[3] Assay Method

Controls	Experiment
Untreated rats. Smears examined after 24 and 36 hr.	Rats treated with the estrogenic ether fraction of bull semen in their vaginas. Smears examined after 24 and 36 hr.
3 rats—no change of the vaginal epithelium.	5 rats—no vaginal epithelial cornification

TABLE 4—Results of Estrogen Assay of Pure Bull Semen Using Allen and Doisy[3] Assay Method

Controls	Experiment
Untreated rats. Smears examined after 24 and 36 hr.	Rats treated with pure bull semen in their vaginas. Smears examined after 24 and 36 hr.
3 rats—no change of the vaginal epithelium.	5 rats—no vaginal epithelial cornification

TABLE 5—Results of Testosterone Assay of the Ether Extract of the Testosterone Fraction of Bull Semen Using Moore et al.[4,5] Assay Method

Controls				Experiment			
Untreated castrated rats				Rats treated with the testosterone ether fraction of bull semen-peanut oil mixture in their right seminal vesicles. Their left s.v. received only peanut oil.			
Rat No.	Body wt. (Gm.)	Wt. of right s.v. (mg.)	Wt. of left s.v. (mg.)	Rat No.	Body wt. (Gm.)	Wt. of right s.v. (mg.)	Wt. of left s.v. (mg.)
1	338.1	148.0	142.6	5	325.6	265.2	136.0
2	320.4	142.3	141.0	6	332.0	246.3	140.8
3	Died	.....	.....	7	319.7	230.8	129.3
4	317.0	139.6	135.7	8	334.1	253.6	142.0
Average		143.3	139.7	Average		248.9	137.0

s.v.—seminal vesicles.

Seventy-two hours after the treatment, all the rats were killed, their seminal vesicles carefully dissected, weighed on a precision balance, then fixed in Boundin's solution, and tissue sections

Fig. 1—Epithelium of seminal vesicle showing tall columnar cells and secretory granules. From a seminal vesicle injected with the ether extract of the testosterone fraction of bull semen. Hematoxylin-eosin stain. x 500.

prepared. These were stained with hematoxylin and eosin.

RESULTS

Estrogen Assay.—Tables 1 and 2 give the results of estrogen assay of the ether extract of the estrogenic fraction of bull semen and of pure bull semen, respectively, expressed in terms of change of rat's uterus weight. It can be seen that no significant differences were found between the uterus weight of the experimental rats and that of the controls, whether using the estrogenic fraction or pure bull semen.

Tables 3 and 4 give the results of estrogen assay of the ether extract of the estrogenic fraction of bull semen and of pure bull semen, respectively, by means of microscopic observation of rat's vaginal

Fig. 2—Epithelium of seminal vesicle showing tall columnar cells and secretory granules. From a seminal vesicle injected with the ether extract of the testosterone fraction of bull semen. Hematoxylin-eosin stain. x 500.

smears. It can be seen that no response (epithelial cornification) was obtained in the treated rats, whether using hormonal ether extract or pure bull semen.

TABLE 6—Results of Testosterone Assay of Pure Bull Semen Using Moore et al.[4,5] Assay Method

Controls				Experiment			
	Untreated castrated rats			Rats treated with pure bull semen in their right seminal vesicles. Their left s.v. received phys. saline sol.			
Rat No.	Body wt. (Gm.)	Wt. of right s.v. (mg.)	Wt. of left s.v. (mg.)	Rat No.	Body wt. (Gm.)	Wt. of right s.v. (mg.)	Wt. of left s.v. (mg.)
1	347.0	144.1	149.8	5	340.5	266.1	147.0
2	351.4	150.0	154.1	6	335.8	258.4	154.5
3	344.6	151.3	148.5	7	354.0	271.6	158.7
4	338.9	146.5	152.0	8	328.9	248.0	136.8
Average		147.9	151.1	Average		261.0	149.2

Testosterone Assay.—Tables 5 and 6 give the results of testosterone assay of the ether extract of the testosterone fraction of bull semen and of pure bull semen, respectively, expressed in terms of increase in weight of seminal vesicles of castrated rats. It can be seen that there is a significant average increase in weight of the seminal vesicles injected with the ether extract of the testosterone fraction of bull semen or with pure bull semen, as compared to the average weight of the seminal vesicles of the same rats which were injected with peanut oil or physiological saline solution. These latter results also hold true when comparing them with the weight of the seminal vesicles of the untreated control rats.

In figures 1, 2, and 3 it can be seen that the epithelium of the seminal vesicles of rats which were injected with the ether extract of the testosterone fraction of bull semen and with pure bull semen, respectively, is tall columnar and contains secretory granules; whereas, in figures 4 and 5, corresponding to the seminal vesicles of rats injected with peanut oil and physiological saline solution, respectively, the epithelium is low cuboidal and deprived of secretory granules.

The findings in these pictures are in agreement with the significant average increase in weight of their respective seminal vesicles from which the microphotographs were made.

DISCUSSION

Estrogen Assay.—The assay methods of Astwood [2] and Allen and Doisy [3] are considered quite sensitive for the detection of estrogens. Using the first method, the assay of minute quantities of estrogens is possible because it is based on the increase in water content of the uterus and thereby increase of uterus weight in immature female albino rats.

With the method of Allen and Doisy,[3] the detection of estrogens by the appearance of cornified epithelium in vaginal smears of spayed, treated mature female albino rats is possible.

However, from the results reported in this paper concerning estrogen assay, it appears that no estrogens were present in the bull semen examined. The absence of estrogens in bull semen is probably a normal occurrence inasmuch as there is no doubt concerning the physical health and fertility of the sources of the semens.

Testosterone Assay.—The assay method of Moore *et al.*[4,5] by increase in weight of and epithelial response of the seminal vesicles of castrated treated rats, offers the most sensitive procedure for the assay of

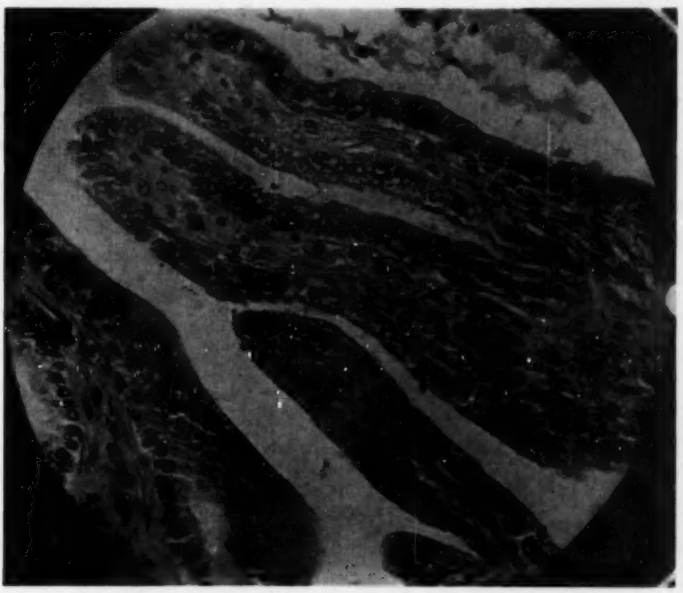

Fig. 3—Epithelium of seminal vesicle showing tall columnar cells and secretory granules. From a seminal vesicle injected with pure bull semen. Hematoxylin-eosin stain. x 500.

testosterone. It is based on the fact that a few days after castration of mature, male albino rats, their seminal vesicles diminish in size and lose the ability to secrete normally; the epithelial cells of these seminal vesicles change from tall columnar to a low cuboidal shape and lose the secretory granules normally present in the distal end of the cells. Both macroscopic and microscopic features of these glands are brought back to normal by the administration of testosterone.

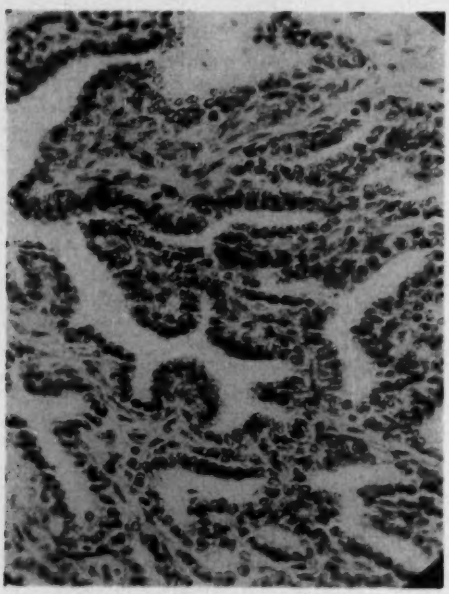

Fig. 4—Epithelium of seminal vesicle showing low cuboidal cells and deprived of secretory granules. From a seminal vesicle injected with peanut oil. Hematoxylin-eosin stain. x 500.

From the results reported herein concerning testosterone assay and from the response of the epithelium of the seminal vesicles as shown on the figures in this paper, it appears that restoration of the macroscopic and microscopic features of the seminal vesicles of the castrated rats occurred after the treatment either with ether extract of the testosterone fraction of bull semen or with pure semen. This undoubtedly indicates the presence of testosterone in the samples of bull semen investigated.

SUMMARY

Two different samples of bull semen were investigated for the presence of estrogens and testosterone. The bio-assays of estrogens using two different methods [2, 3] gave negative results. The bio-assays of testosterone using the method of Moore *et al.*[4, 5] gave positive results.

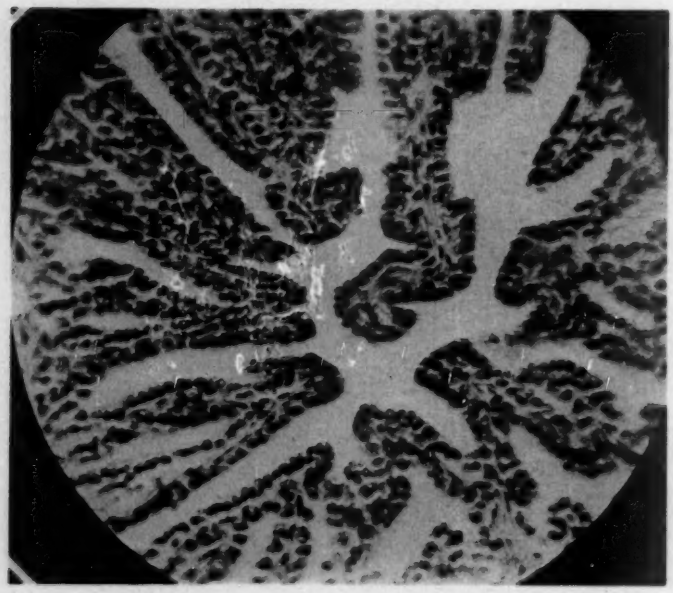

Fig. 5 — Epithelium of seminal vesicle showing low cuboidal cells and deprived of secretory granules. From a seminal vesicle injected with physiological saline solution. Hematoxylin-eosin stain. x 500.

It may be concluded that while the absence of estrogens is probably a normal occurrence in bull semen, the presence of testosterone may play some role in the physiology of reproduction.

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The Effects of Aureomycin and Other Compounds on Horse Strongyle Larvae

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FOR SEVERAL years an anthelmintic screening program has been carried out at this college in which various compounds have been tested for activity against the larvae and eggs of horse strongyles in feces. The present paper reports the results obtained with 14 compounds of various structural formulas.

The technique used has been described by Levine.[3] Briefly, the compound to be tested is diluted with talcum powder to give it body and then mixed with fresh horse feces containing the nematode eggs. The resultant mixture is placed in a cheesecloth bag which is suspended in a glass jar. This is allowed to incubate for a week at room temperature and is then examined by the Baermann technique for larvae. Untreated controls are run in each test. Each compound is usually tested at concentrations of 0.01 M., 0.005 M., 0.0025 M., 0.001 M., and sometimes lower. At the time the aureomycin and hexachlorocyclopentadiene were tested, their molecular weights were not known, so percentile concentrations were used.

RESULTS

Three of the compounds tested were active. Aureomycin prevented the development of larvae in a concentration of 0.00209 M. (0.1 %). This is the first of several antibiotics tested in this program which has shown any anthelmintic activity.

Hexachlorocyclopentadiene prevented the development of larvae in a concentration of 0.0037 M (0.1 %). This is the first chlorinated hydrocarbon tested in this program which had much activity. The *delta* isomer of hexachlorocyclohexane and heptachlor are inactive (*see* below). Levine[2] found that 0.282 M. (10 %) DDT and 0.344 M. (1 %) *gamma* hexachlorocyclohexane had little effect on the eggs and larvae. However, Levine and Ivens[4] found that chlorinated 3-methylpyridine containing approximately four chlorine atoms was active in a concentration of 0.0025 M., (0.058 %) and chlorinated 4-methylpyridine containing approximately five chlorine atoms in a concentration of 0.01 M. (0.265 %). Since pyridine is similar to benzene in many of its properties, the results with these compounds are mentioned here.

The third active compound found in this study was *n*-hexyl isooctyl amine, which prevented the development of larvae in a concentration of 0.0025 M. (0.053 %).

The compounds listed below failed to prevent the development of horse strongyle larvae at a concentration of 0.01 M., the highest concentration tested. In parentheses after each compound is the percentile concentration corresponding to 0.01 M.

Insecticides and Related Compounds

Tetraisopropylpyrophosphate (0.346 %)
Tetrabutylpyrophosphate (0.402 %)
Diethyl phosphoric acid (0.154 %)
delta-Hexachlorocyclohexane (0.291 %)
Heptachlor (1 [or 3a], 4,5,6,7,8,8-heptachlor-3a,4,7,7a-tetrahydro-4,7-methanoindene) (0.373 %)

Anthelmintic

Egressin (thymyl N-isoamylcarbamate) (0.263 %)

Anticoccidial Compounds

Nitrophenide (3,3′-dinitrodiphenyldisulfide) (0.308 %)
Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) (0.198 %)

Other Compounds

2-Nitroresorcinol (0.155 %)
p,p′-Dinitrostilbene (0.270 %)
Pinacolone (0.100 %)

The *delta* isomer of hexachlorocyclohexane is not a good insecticide, but Anderson, Godbey, and Durant[1] reported that 0.0688 M. (2 %) of the compound in water destroyed 99 to 100 per cent of first stage

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The tetraisopropylpyrophosphate, tetrabutylpyrophosphate, diethyl phosphoric acid, heptachlor, pinacolone, and *n*-hexyl isooctyl amine used in this study were furnished by Dr. C. W. Kearns, Department of Entomology, University of Illinois; the 2-nitroresorcinol and p,p′-dinitrostilbene by Dr. C. S. Marvel, Department of Chemistry, University of Illinois; the *delta*-hexachlorocyclohexane by Commercial Solvents Company, Terre Haute, Ind.; the egressin by Sharp and Dohme, Inc., Philadelphia; the nitrophenide by Lederle Laboratories Division, American Cyanamide Company, New York; the nitrofurazone by Drs. Hess and Clark, Ashland, Ohio; and the hexachlorocyclopentadiene by Julius Hyman and Company, Denver, Colo. Appreciation is expressed for their courtesy in making these compounds available for study.

larvae of the kidney worm *(Stephanurus dentatus)*. However, it was inactive in the present study in a concentration of 0.01 M. (0.291 %).

Egressin was introduced as an anthelmintic against the human pinworm, *Enterobius vermicularis*, by Zima *et al.*[6] and Sauer and Weissflug.[5] However, it was inactive against horse strongyle eggs and larvae in feces in a concentration of 0.01 M. (0.263 %).

Summary

Of 14 compounds tested, 0.00209 M. (0.1 %) aureomycin, 0.00307 M. (0.1 %) hexachlorocyclopentadiene, and 0.0025 M. (0.053 %) *n*-hexyl isooctyl amine prevented the development of horse strongyle eggs and/or larvae in feces. Compounds which were ineffective at a concentration of 0.01 M. were tetraisopropylpyrophosphate, tetrabutylpyrophosphate, *delta*-hexachlorocyclohexane, heptachlor, egressin, nitrophenide, nitrofurazone, pinacolone, 2-nitroresorcinol, diethyl phosphoric acid, and p,p'-dinitrostilbene.

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Cross Transmission of Parasitic Worms Between Cattle and Sheep

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THE SUBJECT of cross transmission of helminths between sheep and cattle has had the attention of parasitologists for some time.

Tetley [1] observed that lambs, grazed on pasture previously occupied only by cattle, acquired *Ostertagia ostertagi, Cooperia punctata,* and *Cooperia oncophora,* species of roundworms ordinarily found in cattle. He concluded, as have Hall [2] and Monnig,[3] that cattle would contribute parasites to sheep if these animals were grazed together or followed each other on pastures, although Snell [4] later observed a beneficial effect from grazing the two species together.

Stoll [5] recorded *Haemonchus contortus, Trichostrongylus axei, Cooperia curticei, Capillaria longipes, Trichuris ovis, Nematodirus helvetianus,* and *Gongylonema scutatum* common to both sheep and cattle grazing a new pasture in a period of two years; the small intestinal worms, *Cooperia oncophora* and *Cooperia punctata,* apparently did not transfer to sheep, and *Trichostrongylus colubriformis* did not establish well in cattle. Stoll [6] also reported the transfer of the tapeworm, *Moniezia expansa* from sheep to cattle, and Taylor [7] observed transfer of *T. axei* between sheep and cattle and considered this species better adapted to the latter host.

In studies on calves grazing for six weeks on a pasture previously grazed by parasitized sheep in Queensland, Australia, Roberts [8] found that although calves became infected with *H. contortus, T. colubriformis, T. axei, Strongyloides papillosus, C. curticei, Trichostrongylus parvispiculum,* and *M. expansa,* they exhibited resistance to infection with these species. The species which did not transfer to calves included the lungworm, *Dictyocaulus filaria;* the nodular worm, *Oesophagostomum columbianum;* the hookworm, *Bunostomum trigonocephalum;* the hair worms, *Trichostrongylus rugatus* and *Trichostrongylus vitrinus;* and the medium stomach worm, *Osteragia circumcincta.* This author points out, however, that the last two species have been recorded from cattle in Queensland.

Clunies Ross [9] found that calves were not readily parasitized by larvae of *H. contortus* from sheep, although sheep were easily infected experimentally by larvae cultured from either sheep or cattle.

It is the purpose of this paper to record experiments and observations on the comparative transmission of various worm parasites to calves and lambs grazing together on pastures contaminated by sheep or by cattle.

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MATERIALS AND METHODS

The pastures here designated A and B were between 2½ and 3 acres in size and located on opposite slopes, with about half the area of each on bottom land. They were separated by a ditch and a woven wire fence. During the period covered by these observations, they furnished equal growth of White Dutch clover, Bermuda and carpet grass in the spring, and Bermuda grass and Dallis grass during the summer and early fall.

The calves used were grade Jerseys purchased from local dairies when less than 48 hours old, or dropped by cows at the laboratory. The lambs were of mixed breeding, born at the laboratory, or Southdowns obtained through the coöperation of the Alabama Experiment Station. Unless otherwise noted, all lambs or calves were raised free of parasites in concrete-floored barns until used in experiments.[10] Postmortem examinations to determine the numbers of different species of worms followed the techniques previously described by Porter.[11]

EXPERIMENTAL DATA

Experiment 1.—The first experiment on natural cross transmission was conducted by G. E. Cauthen.* On April 11, 1945, 2 yearling ewes and a 3-month-old lamb, all previously infected experimentally with common stomach worms, *H. contortus,* cultured from sheep, were placed on pasture A. At the same time, 3 6-month-old calves infected with the same species, cultured from cattle, were placed on pasture B. Nine days later 5 para-

* Resigned June 7, 1947.

TABLE 1—Numbers of Stomach Worms, Haemonchus Contortus, Recovered from Calves and Lambs After Grazing Together for Different Intervals on a Sheep Pasture and on a Cattle Pasture (Experiment 1)

Days on pasture	Sheep pasture (A)		Cattle pasture (B)	
	Number of worms recovered		Number of worms recovered	
	calf	lamb	calf	lamb
48	2,012	7,208	2,948	984
54	520	1,720	20,432	16
57	320	1,072	2,488	64
63	104	56	5,360	104
64	40	0	4,200	0

site-free lambs, 2 months old, and 5 parasite-free calves, 6 months old, were added to each pasture as test animals. One calf and 1 lamb from each pasture were slaughtered and examined for stomach worms at the end of 48, 54, 57, 63, and 64 days.

The numbers of worms recovered and other data are recorded in table 1. This table shows that calves acquired many more stomach worms while grazing with infected cattle on pasture B than did the lambs. On pasture A, the lambs acquired more stomach worms from sheep than did the calves. Fewer worms were harbored by lambs after sixty-three and sixty-four days than were harbored by calves at the end of the same periods, indicating the probability of an earlier manifestation of resistance in 2-month-old lambs than in 6-month-old calves.

Experiment 2.—Pasture B was also grazed, following termination of experiment 1, for short intervals by calves until the last of September, 1945, after which it remained vacant until May 17, 1946, when 4 calves, infected with *H. contortus* of bovine origin, were turned on this pasture. At the same time, 2, 5-month-old, parasite-free calves and 2, 4-month-old, parasite-free lambs joined the group as test animals. The test animals were slaughtered on June 28, forty-two days later, and examined for parasites. The 2 calves had 5,728 and 6,624 *H. contortus*, respectively, whereas the 2 lambs harbored only 620 and 14 *H. contortus*, respectively. The calves harbored two and four specimens of *Moniezia benedeni*, respectively, and 1 test calf had two mature *N. helvetianus*, whereas the lambs were free of both these species.

Experiment 3.—Pasture A was grazed similarly to pasture B, after termination of experiment 1, until September, 1945, after which it remained vacant until mid-June, 1946, when 4 parasitized sheep were turned on to graze. These sheep harbored a variety of parasites, including *H. contortus*, *C. curticei*, *S. papillosus*, *T. colubriformis*, *B. trigonocephalum*, *Oc. columbianum* and *M. ex-*

TABLE 2—Numbers of Worms Recovered from Calves and Lambs Grazing on Pasture A in Experiments 3 and 4

	Animal	*Haemonchus contortus*	*Cooperia punctata*	*Cooperia pectinata*	*Cooperia curticei*	*Strongyloides papillosus*	*Bunostomum trigonocephalum*	*Trichostrongylus colubriformis*	*Oesophagostomum columbianum*	*Moniezia expansa*	Other	
Experiment 3, July 1, 1946 — August 13, 1946												
Calf	854	4,320	2,360	160	3,000	0	0	0	0	0		
	857	3,200	2,298	200	3,432	0	0	0	0	0		
	793	2,836	2,394	240	3,046	0	0	0	0	0		
	792	338	340	80	80	0	0	0	0	0		
Total		10,694	7,392	680	9,558	0	0	0	0	0		
Ave.		2,673	1,848	170	2,389	0	0	0	0	0		
Lamb	1	8,288	280	80	3,960	10	0	120	15	18	30	Moniezia sp.*
	2	4,720	320	100	3,720	160	1	160	6	18	18	Moniezia sp.
	5	3,040	120	120	2,000	360	0	0	4	4	31	Moniezia sp.
	6	2,360	105	30	645	120	1	10	3	7	10	Moniezia sp.
Total		18,408	825	330	10,325	650	2	290	28	47	89	Moniezia :
Ave.		4,602	206	82	2,581	162	0.5	72	7	12	22	Moniezia
Experiment 4, October 7, 1946 — November 19, 1946												
Calf	861	15	1,944	160	2,376	0	0	0	0	0	2	*N. helvetianus*
	862	20	1,220	0	300	0	0	0	0	0		
	863	4	3,100	160	3,760	0	0	0	0	0	1	*N helvetianus*
	864	4	700	160	820	0	0	0	0	0	3	*O. radiatum*
Total		43	6,964	480	7,276	0	0	0	0	0		
Ave.		10	1,741	120	1,819	0	0	0	0	0		
Lamb	7	20	200	40	4,040	80	0	40	211	16	144	Moniezia sp.*
	8	180	120	40	3,400	120	0	80	233	0		
Total		200	320	80	7,440	200	0	120	444	16		
Ave.		100	160	40	3,720	100	0	60	222	8		

* Unidentified immature specimens.

pansa. On July first, 4, 4-month-old lambs and 4, 4-month-old calves were added to the pasture. All were parasite-free, except 1 calf which was passing four eggs per gram (e.p.g.) of feces of Cooperia spp., probably *C. punctata* and *C. pectinata*, and 2 of the lambs which were passing a few Strongyloides eggs. These test animals grazed with the parasitized sheep for forty-two days, when they were slaughtered and examined for parasites. The various species of worms acquired by the lambs and calves are given in table 2.

The larger numbers of Haemonchus harbored by the lambs indicated that they were more suitable hosts to this parasite originating in sheep than were the calves. This is further borne out by the fact that 41 per cent of this species in the lambs were mature, whereas only 19 per cent were mature in calves. On an average, there were twice as many *C. pectinata* and nine times as many *C. punctata* in the calves as in the lambs, but the numbers of *C. curticei* in the two hosts were approximately equal. *Bunostomum trigonocephalum*, *T. colubriformis*, *S. papillosus*, *Oe. columbianum*, and *M. expansa* were not transmitted to calves.

Experiment 4.—The 4 parasitized sheep which had been grazing on pasture A during the preceding experiment were removed from pasture on October 7. At this time, 4 calves and 2 lambs were turned on this pasture as test animals. These test animals were approximately 6 months old and had been raised free of parasites. To introduce some cattle parasites other than *H. contortus*, 2 calves carrying moderate numbers of Cooperia spp. and very light infections with Nematodirus and *Oe. radiatum* were added to the group. After grazing the parasite-contaminated pasture until November 19, a period of forty-three days, the test calves and lambs were slaughtered and their parasites recovered.

The results obtained (table 2) generally confirmed the findings in experiment 3, except that the lambs harbored an average of twice as many *C. curticei* as did the calves. *Nematodirus helvetianus* was recovered from only 2 of the calves, and *Oe. radiatum* from 1 calf. These two species were not found in the 2 lambs.

Experiment 5.—Pasture A was vacant from mid-November, 1946, (experiment 4) until April, 1947, when 3 yearling steers and 2, 9-month-old lambs were placed on this pasture. These animals became infected and, in turn, contaminated the pasture. The 2 lambs were removed on May 26 and

TABLE 3—Numbers of Worms Recovered from Calves and Lambs Grazing on Pastures A and B in Experiment 5

	Animal No.	Days on pasture	*Haemonchus contortus*	*Ostertagia ostertagi*	*Cooperia punctata*	*Cooperia pectinata*	*Cooperia curticei*	*Strongyloides papillosus*	*Bunostomum trigonocephalum*	*Nematodirus helvetianus*	*Moniezia expansa*	*Moniezia benedeni*	Other
Pasture A													
Calf	950	35	1,560	30	2,640		60	0*	0	180	22	2	20 Moniezia sp.**
	952	35	2,460	0	11,100	60	240	0*	0	240	18		14 Moniezia sp.
Total			4,020	30	13,740	60	300	0	0	420	40	2	34 Moniezia sp.
Ave.			2,010	15	6,870	30	150	0	0	210	20	1	17 Moniezia sp.
Lamb	13	35	90	0	390	0	60	2,040	105	0	3	0	2 Moniezia sp.
	14	35	210	0	90	0	300	60	0	0	11	0	20 Moniezia sp.
Total			306	0	480	0	360	2,100	105	0	14	0	22 Moniezia sp.
Ave.			153	0	240	0	180	1,050	52	0	7	0	11 Moniezia sp.
Pasture B													
Calf	948	19	1,056	360	17,520	0	0	0	0	800	0	1	
	949	35	630	1,100	36,240	0	0	0*	0	2,040	0	1	
Total			1,696	1,460	53,760	0	0	0	0	2,840	0	2	
Ave.			848	730	26,880	0	0	0	0	1,420	0	1	
Lamb	11	19	60	0	312	0	0	0*	0	0	0	0	
	12	35	240	0	4,740	0	0	0*	0	0	0	0	
Total			300	0	5,052	0	0	0	0	0	0	0	
Ave.			150	0	2,526	0	0	0	0	0	0	0	

* Eggs seen in feces eighteenth and twentieth day. ** Unidentified immature specimens.

returned to a separate pasture. The 3 infected steers remained on pasture and were joined on June 5 by 2, 4-month-old calves and 2, 4-month-old lambs.

Pasture B had carried 2 to 6 parasitized yearling steers from the conclusion of experiment 2 (June 28, 1946), varying with the amount of forage available. Hay and grain was fed from December, 1946, through March, 1947. Three infected yearlings were on pasture when 2, 4-month-old lambs and 2, 4-month-old calves were added as test animals on June 5. At this time, there was an abundant growth of white clover on both pastures A and B.

One calf and 1 lamb on pasture B were slaughtered at the end of nineteen days. The other calf and lamb on pasture B and both calves and both lambs on pasture A were slaughtered after thirty-five days on pasture. The worms recovered are listed in table 3.

Haemonchus contortus was apparently better adapted to calves than lambs on pasture A, perhaps due to the passage of successive generations through the calves on the pasture in experiments 3 and 4 and the calves immediately preceding these test animals. On pasture B, however, where contamination from cattle had always predominated, this species still established better in calves than in lambs.

As in previous experiments, *C. punctata* developed much better in calves than in lambs on both pastures. On pasture A, *C. curticei* developed equally well in both hosts, whereas *C. pectinata* was present in small numbers in the 2 calves but absent in the lambs. Where present, *B. trigonocephalum* developed in 1 of 2 lambs but not in calves, and *N. helvetianus*, *O. ostertagi*, and *M. benedeni* developed in calves but not in lambs. The tapeworm *M. expansa* developed in both calves and lambs on pasture A but *M. benedeni* was not found in lambs on either pasture. Very light infections with *S. papillosus* appeared in the calves on both pastures but were eliminated before necropsy. Very light infections were established in lambs on pasture B, whereas these parasites were established in sufficient numbers to be recovered from the lambs on pasture A at the end of thirty-five days.

Subsequent Observations

Pasture A was grazed by 2 to 4 calves or yearlings at intervals after the termination of experiment 5 on July 10, 1947, which was the last date sheep were on this pasture. The parasites present on the pasture were determined by recovery of parasites from 5 animals grazing thereon since that time. These consisted of a 6-month-old calf on the pasture in January and February, 1948, a similar calf, July and August, 1949, and 3 yearlings which grazed the pasture in August and September, 1950. The species recovered and the numbers of animals harboring the various species were as follows: *H. contortus*, 3; *O. ostertagi*, 4; *C. punctata*, 5; *N. helvetianus*, 3; *O. radiatum*, 4; and *M. benedeni*, 2. It is interesting to note that certain species present in fair number in calves in the earlier experiments, namely, *C. curticei*, *C. pectinata*, and *M. expansa*, were not encountered.

Summary

1) Calves grazing on pastures contaminated by cattle acquired appreciably more stomach worms *(Haemonchus contortus)* than did lambs grazing together on the same pasture.

2) In similar tests with this species, when lambs and calves followed sheep, the lambs acquired appreciably more of these parasites than did calves.

3) Continued contamination of a pasture by cattle infected with *H. contortus* from both sheep and cattle resulted in test calves eventually acquiring more stomach worms than did lambs during a comparable grazing period.

4) The small intestinal worms from cattle, *Cooperia punctata* and *Cooperia pectinata*, developed in numbers ranging from 9 to 30 times and 2 to 30 times greater, respectively, in calves than in lambs, whereas *Cooperia curticei* from sheep developed approximately as well in calves as in lambs.

5) Although the threadworm, *Strongyloides papillosus*, and the small hair worm, *Trichostrongylus colubriformis*, occur in young cattle in Alabama, the former species was transmitted poorly, and the latter species was not transmitted at all to calves exposed to these parasites from sheep.

6) Of the tapeworms, *Moniezia expansa* was acquired by both hosts, but *Moniezia benedeni* was not found in lambs.

7) In these experiments, the nodular worms, *Oesophagostomum radiatum*, thread-necked strongyles, *Nematodirus helvetianus*, and the medium stomach worms *Ostertagia ostertagi*, which are parasites of cattle, were not transmitted to lambs. Similarly, the nodular worms, *Oesophagostomum columbianum*, and hookworms, *Bunostomum trigonocephalum*, parasites of sheep, were not transmitted to calves.

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Physiological Changes in the Mammalian Pineal Gland Correlated with the Reproductive System

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ALTHOUGH the pineal gland has interested scientific investigators from the times of the ancient Greeks and Romans, it was only during the first three decades of this century that a tremendous amount of research and experimental work was done in an effort to clarify what might be the physiological function of this organ. Nevertheless, after reviewing the literature on the work that was done in studying the pineal gland we still can quote Laignel-Lavastine [28] who wrote, "Nothing, or almost nothing, is known of the physiological significance of the pineal gland."

There exists notable disagreement in the results obtained by the investigators regarding the anatomy, morphology, histology, pathology, and physiological function of the pineal body. From the standpoint of comparative anatomy, the pineal gland is considered by the majority of the authors as a rudimentary representative of the third eye of the Lacertidae without any actual physiological function in mammals. According to Fuchs,[29] the embryology of the parietal organ in some lower reptiles indicates clearly that this body is a remnant of a third eye which still had retained some of its controlling power upon pigmentation. This conception is supported by the fact that after feeding pineal tissue to 10-day-old larval forms of tadpoles, pigment changes were always evident.[31] Bors and Ralston [4] reported that *Xenopus laevis* exhibits a contraction of melanophores after the injection of pineal extracts. Gladstone and Wakeley [20] mention the existence of the pineal organ in some of the most ancient types of fossil fish, but they state that it is evident that this organ in these extinct fishes had already retrogressed to a vestigial stage. The parietal organ, having lost its connection with the brain and also with the source of light, obviously ceased to function, at least as a light-perception organ, in this period in the evolution of the vertebrates.

The histological conception of this organ has been a most controversial point among investigators. According to Rio-Hortega,[37, 38] there are not two histologists who give the same interpretation of the nature and morphology of the pineal gland. The reports presented by pathologists and clinicians in relation to the function of the pineal gland are no less contradictory than those presented by anatomists and histologists. Such is the case of Pellizzi's macrogenitosoma praecox,[35] which has been regarded as a consequence of pineal disturbances. Macrogenitosoma praecox has been reported in about 50 per cent of the patients without any disturbance of the pineal structure and vice versa. Haldeman,[26] who reviewed a great many reports of pineal tumors, said that the majority of the patients did not exhibit sexual syndromes.

It is widely believed that the pineal gland reaches maturation in man at the age of 6 or 7 years. Thereafter, the appearance of involutive phenomena, according to Globus and Silber,[25] may be cyst formation, deposits of calcium, hyalinization, and fibriles. However, Rio-Hortega [38] pointed out that the loss of the function of some of the cellular components does not mean the disappearance of all activity in the remaining elements. He demonstrated that the pineal gland of 10-year-old children very often presents the same characteristics as those of adult pineals. This author's opinion is that "the pineal performs other postpuberty functions of a permanent character but perhaps less active," in addition to prepuberty function. According to Trautmann,[42] the structure of the pineal gland in cattle does not undergo physiological involution after puberty.

In regard to the calcification of the pineal gland, Gaskel,[21] Marburg,[30] Lilja,[27] McNeal,[33] and others claim that calcification of the pineal gland increases gradually with age. But McNeal stated that the process of calcification does not necessarily mean that the phenomenon of calcification is a degenerative process which affects the whole physiological function because, while one pineal lobule becomes exhausted the remaining ones take over their function. Gignolini [22] reported 34 cases with signs of calcification in 210 cranial roentgenograms that he performed. On the other hand, Gladstone and Wakeley,[24] in 300 adult skulls over 16 years of age, found the pineal gland to be calcified in the proportion of 65 per cent.

Some authors attempted to determine whether any relationship exists between the pineal gland

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and pregnancy. Brandenburg[5] reported some differences in shape of the gland in women due to the number of pregnancies. Frada and Micale[20] made a comparative study of the function of the gland during pregnancy and its activity in nonpregnant women. Three groups of 40 women each were studied with the following results: The pregnant group showed 41 per cent of the pineals with appreciable concretions; the group of women with a previous pregnancy presented concretions in 40 per cent of the cases; and finally, the group of women who had never been pregnant exhibited pineal concretions in 15 per cent of the cases. These authors claim that one pregnancy seems to be enough to produce concretions. It was reported by Desogus[12] that the bitch pineal gland during pregnancy underwent physiological involution which became more noticeable during the parturition and puerperium.

The correlation of the pineal gland with the gonads has already been studied by Binch and Hulles[2] who observed that the pineal organ of the ox was smaller than that of the bull. Also, these authors found atrophy of the parenchyma of the pineal of a cat which had been castrated when about 7 months old. Aschner[3] and Calvet[4] arrived at the same conclusion regarding the weight of the pineal in castrated and normal animals. Both authors reported important changes in the histological picture of the pineal gland in castrated dogs, cats, rabbits, guinea pigs, and oxen. Von Kup[43] found that castration in pigs before they reach sexual maturity causes pineal hypertrophy. The influence of sexual activity upon the pineal gland has been reported by Clemente[7,8] and Desogus[13,14] in chickens and dogs. These investigators have found a remarkable relationship between the state of the pineal gland and the gonadal condition. Quinlan[30] and DeLange[11] fail to find evidences of change in weight, macroscopic and histological appearance of the pineal gland in sterile cattle.

The results obtained by destroying the pineal gland *in situ* by cautery and pinealectomy supported the criterion of an endocrine function related to the development of the sex glands. On the other hand, these results have been entirely discredited by similar experimental work conducted by other authors. The experimental work dealing with feeding, administration of pineal extracts or grafts, has also been reported with the same conflicting results. In brief, all methods applied in the identification of the endocrine function in other glands have been exhausted in dealing with the pineal without consistent results. For this reason, during the last twenty years the pineal gland has been almost forgotten in the English literature; the majority of scientists mention the pineal gland in the classroom only as a curiosity in the history of endocrinology.

This manuscript attempts to add some additional information to the classic works of Flesch,[18] Nicolas,[33] Dimitrova,[16] Favaro,[17] Constantini,[9] Jordan,[27] and Amprino[1] which deal with the embryology, morphology, and histogenesis of the pineal gland in sheep and cattle. The data to be presented emphasize the morphological variations of the pineal gland of sheep, cattle, and rats related with the physiological condition of the reproductive organs.

MATERIALS AND METHODS

The material for this investigation consisted of 153 pineal glands from sheep, 110 from cattle, and 755 from rats. The pineal glands from sheep and cattle were obtained from a local meat-packing company. All glands from pregnant sheep were obtained in the veterinary clinic after the animals had been used for the purpose of training in surgery. The glands were collected as soon as possible after the animals were slaughtered and then placed immediately into small vials and brought to the laboratory on ice. The weight of each gland was determined on an analytical balance. The white rats were obtained from the Maxfield Animal Supply Company, Montgomery, Ohio. Some adult rats were obtained from the colony maintained by the College of Medicine.

Material for histological study was fixed in Bouin's fluid or 10 per cent formalin. Delafield's hematoxylin and eosin were found to be satisfactory stains.

RESULTS

THE PINEAL GLAND IN SHEEP

In general, the pineal gland in sheep is round to ovoid in shape and varies greatly in size among individuals. The size and weight of this gland appears to be related to the physiological state of the reproductive system rather than to the breed, age, or size of the animal (fig. 1).

The Pineal Gland in Nonpregnant Sheep.—The mean weight of the pineal glands in this entire group of 50 sheep was 81.34 ± 27.29 mg. The range in weight varied from 43 mg. to 149 mg. When the weights were determined for each age group, it was found that the 1-year-old nonpregnant females had the smallest pineal gland weight, 56.50 ± 12.30 mg. The 2-year-old nonpregnant females had the largest mean weight, 95.40 ± 27.32 mg. The 3-year-old group showed the greatest variation in pineal gland weight, 78.33 ± 44.27 mg. The pineal glands in the 4-year-

old group weighed 86.00 ± 18.38 mg.; that is, they weighed more than the 3-year-olds but less than those of the 2-year-old group. Since only 22 sheep were obtained for the 4-, 6-, 7-, 8-, 9-, and 10-year-old nonpregnant female groups, they were grouped as one with the glands weighing 79.10 ± 25.82 mg.

It is interesting to note that approximately 25 per cent of the females studied possessed pineal glands weighing in the 40- and 50-mg. divisions whereas another 25 per cent had glands weighing from 110 to 149 mg. Approximately 10 per cent of the sheep had glands weighing near the mean weight for the group as a whole (81.34 ± 27.29 mg.).

Attempts were made to establish a possible relationship between the pineal gland weights and the physiological state of the reproductive system. The ovaries of those animals studied were in a quiescent or anestrous state. The glands and ovaries studied were collected from January through April, 1952.

The Pineal Gland in Pregnant Sheep.—The pineal gland in pregnant sheep presents evidence of increased function when compared with that in nonpregnant ewes in that it shows increased vascularity, perivascular lymph spaces, increased size and weight. In a total of 24 pineal glands from pregnant ewes, 75 per cent weighed more than the mean weight of pineal glands from nonpregnant ewes.

The pineal gland weights in this group ranged from 46.2 to 253 mg. Mean weight for the 3-year-old pregnant ewe group was 98.73 ± 11.21 mg. The mean weight for the 4-year-old group was 84.76 ± 20.61 mg. and for the 5-year-old group 93.20 ± 18.89 mg. The gland from a 7-year-old pregnant ewe weighed 163 mg. and that of a 10-year-old pregnant ewe weighed 253 mg. The mean gland weight for the entire pregnant ewe group was 101.89 ± 39.04 mg. and is greater than the mean gland weight (81.34 ± 27.29 mg.) of the nonpregnant group.

In order to make the pregnant and nonpregnant ewe groups comparable in age, the weights of the pineal glands were determined for the nonpregnant group by omitting the 1- and 2-year-old sheep. When this is done, the mean weight of the glands from the nonpregnant group is 81.11 mg.

The Pineal Gland in Normal Male

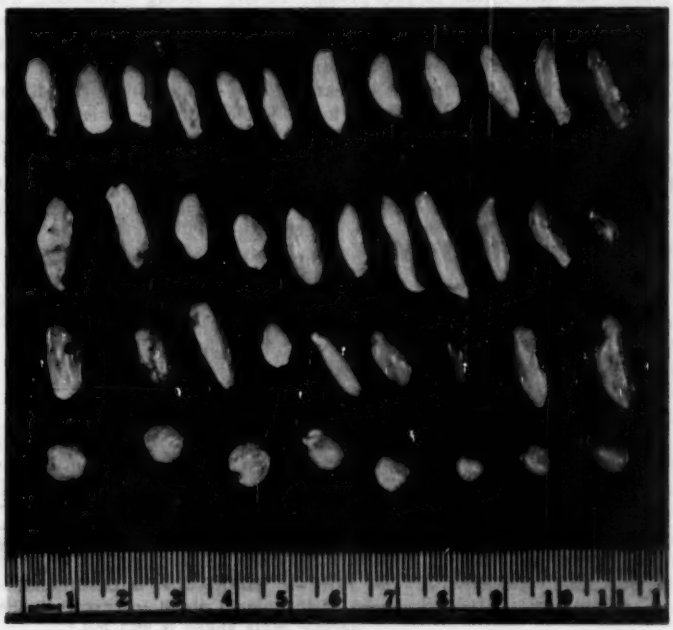


Fig. 1—Pineal glands from cattle and sheep. Reading from the top down, the first and second rows are pineal glands from bulls 1 to 6 years of age; the third row, from cows 1 to 8 years of age; the fourth row, from male sheep—the last three are glands from castrated animals.

Sheep.—The pineal glands in this group of sheep were collected at the same time as those of the nonpregnant female ewe group (January through April, 1952). Glands were obtained from 20 rams, the weights ranged from 39.4 to 204 mg. The mean for the entire group was 96.14 ± 38.58 mg. The glands from 2-year-old rams weighed 96.64 ± 27.07 mg., while the mean weight of the glands from 3-year-old rams was 102.96 ± 40.28 mg. The weight for glands from the 4-year-old rams was 87.37 ± 45.78 mg. The mean pineal gland weight for these three groups showed marked variation. The glands of lightest weight were found in the 4-year-old ram group.

Also observed among the 4-year-old rams were many glands with extensive areas of melanin infiltration. The glands from the 2- and 3-year-old rams appeared active and showed no evidence of atrophy.

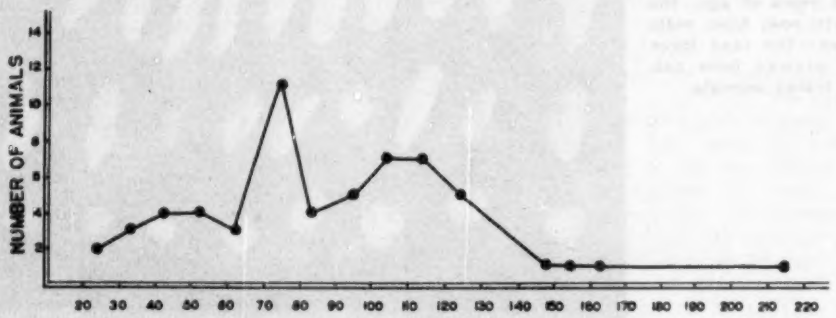
The Pineal Gland in Castrated Male Sheep.—The pineal glands in the castrated male sheep ranged in weight from 22 to 214 mg. The mean weight for the total of 59 glands was 87.74 ± 36.38 mg. The mean gland weight for the 1-year-old castrated sheep was 72.81 ± 28.84 mg. The smallest gland was also found in this group. In the 2-year-old castrated group the mean weight was found to be 101.90 ± 40.38 mg. The heaviest and most consistent gland weights were found in the 3-year-old castrated group, 103.32 ± 15.24 mg. Since only ten of the 59 glands studied were found in the 4- to 11-year-old groups, they were combined as one group with a mean weight of 64.26 ± 23.25 mg.

When the gland weights of this entire group of castrated sheep are graphically represented, it will be found that the largest number of individuals fall between 60 and 80 mg. Between 22 and 60 mg., the largest number of individuals had glands weighing 40 mg. Another group of rather uniform gland weights was found between 80 and 129 mg. In addition, four heavier glands were found weighing 149, 155, 162, and 214 mg. respectively (graph 1).

THE PINEAL GLAND IN CATTLE

The anatomical form of the pineal gland in cattle varies markedly with age (fig. 1 and 2). In young animals, it is somewhat cylindrical in outline and varies in length. In old cattle, the gland is cone-shaped, pyriform, or spindle-shaped.

The Pineal Gland in Bovine Females.—The pineal glands from 80 heifers and cows ranging in age from 5 months to 11 years were studied. Mean weight was determined on 10 Jersey and Guernsey 5-month-old calves and found to be 78.50 ± 32.38 mg. A gradual increase in size and weight was noted in the glands from calves 12 to 24 months of age, the mean weight being 133.80 ± 24.60 mg. The pineal glands from a 2-year-old group of heifers weighed 161.69 ± 39.58 mg. In the 3- and 4-year-old cows the pineal glands attain maximum development. The mean weight of the 3-year-old group was 274.50 ± 78.27 mg., and the 4-year-old group, 261.37 ± 95.77 mg. The mean weights of the 5-year-old group was 262.00 ± 88.63 mg. Macroscopically, at this time, there appears some



Graph 1—Variation in weight of the pineal gland in castrated sheep. (The average in normal male sheep is 96.1 mg.)

evidence of beginning atrophy, especially of the free end of the gland. The 6-year-old cows have glands whose mean weight is less (193.66 ± 62.53 mg.) than the 5-year-old group. The mean gland weight for the 7-year-old cows was 178.00 ± 46.69 mg., for the 8-year-old cows, 229.27 ± 73.92 mg., and for the 10- to 11-year-old cows 238.00 ± 72.83 mg. In these older animals, many glands were found with melantic infiltration. It should be pointed out that glands will be found among 8- and 10-year-old cows that appear as active as pineals from 3-year-old animals.

It is interesting to note that glands from 14 beef cows were studied and the maximum weight for a gland in this group was 116 mg. less than the maximum weight gland in the dairy breeds. Only 4 beef cows had glands weighing over 200 mg., yet the ages ranged from 2 to 6 years.

The Pineal Gland in Bulls.—There is much variation in the size and weight of the pineal gland in bull calves during the early months of postnatal life. The mean weight was determined on 13, 5-month-old bull calves and found to be 79.69 ± 37.61 mg. Another group of glands from 6- to 18-month-old bull calves weighed 160.50 ± 33.90 mg. The mean weight was determined on only 5 adult bulls ranging in age from 2 to 6 years and was found to be 174.00 ± 53.71 mg. The pineal gland from 1, 10-year-old Holstein-Friesian bull weighed 318 mg.

The Pineal Gland in Castrated Bovine Males.—The pineal glands from 10 castrated male calves ranging in age from 7 to 16 months of age showed less variation in size than most of the groups studied. The mean weight was 96.50 ± 21.45 mg. Ten castrated adults ranging in age from 2 to 5 years were studied and showed a mean weight of 160.50 ± 74.91 mg. The range in individual gland weights was from 98 to 325 mg.

THE PINEAL GLAND IN RATS

The rat pineal gland is a globular or pear-shaped structure lying on the surface of the brain between the cerebrum and the cerebellum.

In a study of 425 adult male rats, the average weight was 0.9 mg., ranging from 0.2 to 1.9 mg. In 250 adult female rats, the average weight was 0.7 mg., ranging from 0.3 to 1.4 mg. An average weight of 0.2 mg., ranging from 0.05 to 0.7 mg., was found in 80 immature rats, both male and female, from 25 to 30 days of age.

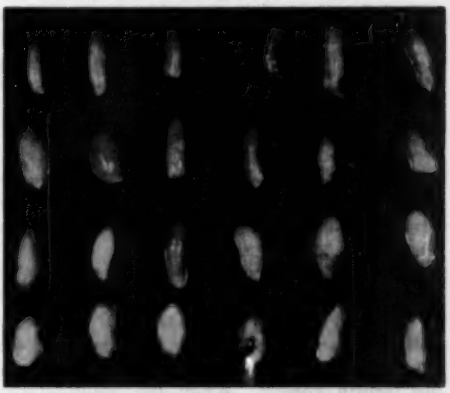


Fig. 2—Pineal glands from dairy cows. Reading from the top down, the first and second rows are pineal glands from Holstein-Friesian cows 1 to 7 years of age; the first three glands of the first row, from heifers 1 to 2 years of age, and the last two glands of the second row, from cows 7 years of age; third and fourth rows are pineal glands from Guernsey cows arranged in order of age from 1 to 7 years.

DISCUSSION

The pineal gland in sheep varies greatly in weight and size. This variation appears to be related to the physiological state of the reproductive system. Physiological evidence has been obtained in this investigation by studying pineal glands from sheep within the following classification: (a) glands from nonpregnant sheep; (b) glands from pregnant sheep; (c) glands from normal female and male sheep; and (d) glands from castrated male sheep.

The pineal gland weight in the group of nonpregnant sheep ranged from 43 to 149 mg., the mean for the entire group being 81.34 ± 27.29 mg. When the weights of the glands are graphically presented, 25 per cent weighed from 40 to 50 mg., whereas another 25 per cent weighed 110 to 149 mg. At present there seems to be no explanation for this distribution.

Attempts were made to disclose a possible relationship between the physiological state of the reproductive tract and the physiological variation in the size and

weight of the pineal gland. This was possible in only a few cases. The largest glands were observed in ewes whose ovaries exhibited well-developed ovarian follicles. The smallest glands were found in ewes whose ovaries were in an anestrous state. The small number of animals studied does not permit definite conclusions but does establish interest for future investigations.

It must be kept in mind that the pineal glands from this group of sheep were collected when normally they should have been pregnant or lambing. The question arises as to why these animals were sent to slaughter. Did they fail to conceive? What can be the relationship between the hypo- or hyperfunction of the pineal gland to sterility?

The pineal gland in pregnant sheep showed marked vascularity and perivascular lymph spaces. When the weights are compared with those of the nonpregnant group they are greater. The pineal gland weights for this group ranged from 46.2 to 253 mg. When weights of the 24 pineal glands studied in this group are compared with those of the nonpregnant group, it is found that 75 per cent weighed more than the mean (81.34 ± 27.29 mg.). The mean weight for the glands from the pregnant ewes studied was 101.89 ± 39.04 mg.

On the basis of these limited observations, it appears that the pineal gland hypertrophies during pregnancy in sheep. What is the function of the pineal gland in pregnancy? Future research will disclose the answer.

A comparative study of the pineal function in pregnant and nonpregnant women has been made by Frada and Micale.[19] These authors claim that the concretions in the pineal gland increase with each pregnancy. For this reason, in the authors' opinion, the endocrine function of the pineal gland undergoes hypofunction and involution while the pituitary gland is in physiological hyperfunction. The occurrence of partial calcification does not necessarily mean hypofunction of the gland. McNeal [32] stated that when one pineal lobule becomes exhausted the remaining ones take over the function. Such is the case of the ovarian mechanism which continues to be an active organ until the total ovary becomes sclerotic. Moreover, the phenomenon of calcification itself may mean the termination of a high metabolic process. Although the introduction of cytochemical observations was not intended at this time, it is interesting to note that some pineal glands from pregnant ewes studied for alkaline phosphatase, showed a high degree of alkaline phosphatase in the endothelial lining of the extensive capillary networks. A similar reaction was noted in the pineal of a 4-month-old sheep fetus.

The pineal gland in normal male sheep between 2 and 3 years of age did not present signs of atrophy. In rams of 4 and 5 years of age, glands were found with extensive areas of melanin infiltration. The three smallest glands in this group of animals were from rams 4 and 5 years of age. Based upon size, weight, and histological data, it would appear that in the second year of life the pineal gland in the ram reaches its fullest development and commences to undergo atrophic processes between the fourth and fifth year of development. Three pineal glands weighing 130, 157, and 207 mg. respectively were very hypertrophic. This might be explained by the fact that individual factors are involved in regulating seasonal involution. Since the glands were collected in different months of the year, this would be sufficient to explain the variation in weights.

The pineal gland weights in castrated male sheep ranged from 22 to 214 mg. The largest glands were found in the 2- and 3-year-old castrated sheep. The mean weight for the entire group was less than that for the normal males (87.74 ± 36.38 versus 96.14 ± 38.58 mg.). Histological evidence was obtained that showed a large number of glands from 1- and 2-year-old castrated sheep with signs of atrophy. On the other hand, a few glands were found to be greatly hypertrophied. The meaning of this variation can not be established at the present time. It can be postulated that the time from castration to slaughter would determine to a great extent how much atrophy had taken place. Recently castrated males should have larger pineal glands than those castrated one year prior to slaughter. It should be recalled that the figures presented by Cutare [10] in regard to the weight of the pineal gland in sheep are in every instance 100 per cent higher than the weights found in this work. Perhaps more important than Cutare's figures for the pineal weights are the results achieved

in this investigation, in relation to Jordan's [27] statement that the entire pineal gland in sheep decreases in size after the first year. This author emphasizes the belief that if the pineal gland has any important function in sheep, this function must be demonstrated only until the eighth month of life. This statement has been discredited by other authors and also by the results of the present investigations. In regard to the weight of the pineal gland in sheep and cattle, the results reported here are in accordance with Streeter.[41]

There is a gradual increase in the weight of pineal glands from 5-month-old to 24-month-old bovine females. The gland appears to reach fullest development in the 3-year-old group of female cattle. After the fifth year, a degenerative process begins to appear, although, in many cases, pineal glands were found in cows of 8 to 10 years of age with the same aspect of an active organ as in 3-year-old females. From the limited data studied to date, it appears that dairy cows have a more highly developed pineal gland than beef cows. Figure 2 illustrates the evolution of the pineal gland in dairy cattle. How far this gland can be related to the phenomenon of milk secretion is not known. The only reference found in the literature relating to this point was that of Davis and Martin [11] who mention "inadequate lactation in pinealectomized cats."

The pineal gland in bull calves shows the same rhythmic development as in female calves. There is a marked variation in size during the early months of postnatal life and later. In order to illustrate the effect of castration in young calves on the size of the pineal gland, it should be recalled that the gland in the normal bull calf group had a mean weight of 160.50 ± 33.90 mg., while the castrated group of the same age had a mean gland weight of 96.50 ± 21.45 mg.

In adult bovine males, castration produces the same effect upon the pineal gland as it does in adult castrated male sheep. An enormous variation in size was found; the smallest gland weighed 98 mg. while the largest weighed 325 mg. Unfortunately, the length of time from castration to slaughter could not be recorded. Nevertheless, the relationship of the testis to the pineal gland has become evident. As early as 1912, Biach and Hulles [3] observed that the pineal gland of oxen was smaller than that of the uncastrated male. These authors also found atrophy of the parenchymatous cells in cats which had been castrated when very young. Results contradictory to these were reported by Ruggeri [40] and Pelligrini.[34] Moreover, Aschner [2] pointed out that in the pineal gland of steers, castrated guinea pigs, rabbits, dogs, and cats, the neuroglia cells are more abundant than the parenchymatous elements. All of the above results were supported by Calvet,[6] who also reported that neuroglia cells are the main element in the pineal gland of the steer while that of the bull contains more parenchymatous cells.

These reports regarding the loss of parenchymatous tissue in the pineal gland after castration are important. According to Rio-Hortega,[37-39] the parenchymal cells of the pineal gland are the more important secretory elements of that gland, although neuroglia cells, as shown by this author, also present some evidence of secretory activity.

All of these statements lend strong support to the theory that the pineal gland should be included among the glands of internal secretion.

The pineal gland in immature rats was so small (0.5 mg. was the average weight in 80 rats) that, in experimental animals, it was not possible to determine any conclusions from the weights. However, by using a large number of adult animals, it was found that in males the pineal gland is larger than in females. The differences in weight between sexes were also observed in sheep and cattle. The interpretation of this fact may be related to the physiological state of the reproductive system. In males, there is more or less permanent sexual activity, whereas females exhibit rhythmic sexual cycles which may be reflected in the pineal gland.

SUMMARY

1) This investigation consisted of a study of 153 pineal glands from sheep, 110 from cattle, and 755 from rats in an attempt to correlate physiological changes in the gland with the reproductive system.

2) The mean pineal gland weights for various age groups are reported for nonpregnant and pregnant ewes, rams, and castrated male sheep; calves, nonpregnant

cows, bulls, and steers; and adult and immature white rats.

3) In nonpregnant ewes, the largest pineal glands were found in those individuals exhibiting well-developed ovarian follicles.

4) The mean pineal gland weight in pregnant ewes is greater than in nonpregnant ewes.

5) The mean pineal gland weight in castrated male sheep is less than in the ram.

6) The pineal gland appears to be larger in the dairy cow than in the beef cow.

7) Castration causes the pineal gland of the steer to weigh less than the gland in the bull of similar age.

8) The pineal gland in the male is heavier than in the female.

9) The possible significance of these observations is discussed.

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Observations on the Lethal Action of Polyborate on Swine Kidney Worm (*Stephanurus Dentatus*) Larvae in Soil

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FROM THE economic point of view, kidney worm infection represents one of the most important swine parasite problems.

Spindler[1] has estimated that in the southern part of the United States losses from trimming of infected carcasses and condemnation of livers and kidneys amount to more than 2.7 million dollars annually. Anderson and co-workers[2] showed that pigs infected experimentally with kidney worms did not gain as much weight as the noninfected control animals. In their experiments, pigs which were infected at weaning age averaged 170 lb. at slaughter, whereas the control animals averaged 235 lb. It was estimated that if these noninfected pigs received approximately 6 lb. of grain ration per day at 4 cents per pound, it would have cost an additional $14.16 for the infected animals to attain a weight of 235 lb. In Hawaii, as well as in many areas of the Pacific,[3] kidney worms of swine are widespread and are recognized as a problem of considerable economic interest (fig. 1). According to observations by Dr. J. M. Gooch, veterinarian for the Hawaii Department of Health, among 16,323 pigs slaughtered on the Island of Oahu from January to April, 1952, 3,466 (21.2%) had kidney worms. Furthermore, data[4] obtained from one of the Honolulu slaughterhouses indicated that during 1947 livers valued at about $13,000, 11 per cent of pigs slaughtered, were condemned because of parasitism due largely to kidney worms.

At the present time, no drug is known which is effective against these parasites. Control of the infection in swine, therefore, must rest on the utilization of proper preventive measures. In this connection, and on the basis of information on the location and development of kidney worm larvae on pastures, investigators[5] of the U. S. Bureau of Animal Industry have suggested a plan of management for sows and their litters. This plan recommends the provision of a bare strip around the pasture so that kidney worm eggs deposited there with the urine will perish from exposure to sunlight. Clunies Ross and Kauzal[6] reported that spraying soil with 10 per cent copper sulphate or 5 per cent creosol, at the rate of 10 gal. per 100 square yards, was effective in killing superficially located kidney worm larvae. Spindler,[7] however, reported that 1 per cent copper sulphate applied at the rate of 15 cc. per sq. ft. was ineffective against these larvae. More recently, Anderson and co-workers[8] have shown that commercial benzene hexachloride and the *delta* and *epsilon* isomers are effective *in vitro* in destroying the preinfective kidney worm larvae. No data is available at this time on the efficacy of these products on larvae present in soil.

Fig. 1—Sow heavily infected with kidney worms. The urine eliminated by this sow in two mornings contained approximately 30,000 and 42,000 kidney worm eggs, respectively.

Because of the need for improved methods in the destruction of kidney worm larvae in soil, observations have been conducted by the writer to determine whether polyborate would be effective. This study was based on recent reports by Hoerlein[9,10] that various boron products were found effective in destroying the larvae of the dog hookworm (*Ancylostoma caninum*) in soil. Hoerlein recommended use of the sodium borate product "borascu" ($Na_2B_4O_7 \cdot {}_4H_2O$) in dog runs with dirt floors, at the rate of 10 lb. per 100 sq. ft.

Based on the studies conducted by Hoerlein, a commercial concern* now has a product marketed under the trade name "polybor-3" (herein referred to as "polyborate") which is intended for use on dog runs to control hookworm larvae. This product is a fine, readily soluble, granular salt, consisting of sodium pentaborate tetrahydrate ($Na_2B_{10}O \cdot 6 \cdot {}_4H_2O$), 77 per cent, sodium tetraborate pentahydrate ($Na_2B_4O_7 \cdot {}_5H_2O$), 18 per cent, and inert ingredients, 5 per cent. The manufacturers suggest that it be applied either in dry

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* Pacific Coast Borax Co.

form at the rate of 5 lb. per 100 sq. ft. or sprayed at the rate of 3 gal. (containing 4.5 lb. of the powder) per 100 sq. ft. This product kills vegetation and should not be used where vegetation is to be maintained.

The purpose of this paper is to report observations relative to the effect of polyborate on the eggs and on preinfective and infective kidney worm larvae in experimentally infected soil plots.

MATERIALS AND METHODS

This study was designed to ascertain the number of viable kidney worm larvae, and/or their infective power, following exposure to soil which was treated with polyborate. In carrying out this plan, preliminary tests were performed indoors with sterilized infected soil in small receptacles. Later the tests were performed outdoors under simulated natural environment, using small soil plots. The type of soil used for all these experiments was a volcanic, silty clay which was fairly porous. For the indoor experiments, square celluloid receptacles were used, each having a surface area of 1/30 of a sq. ft. (2.19 inches square) and approximately 11/16 in. high (fig. 2). Thirty-five

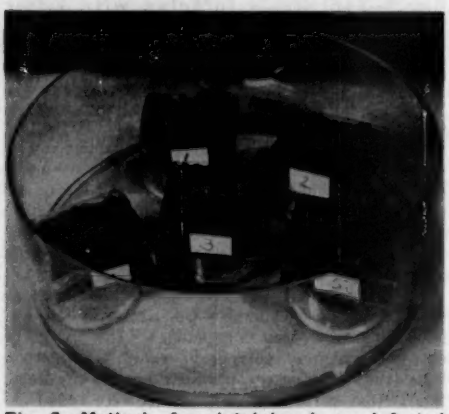

Fig. 2—Method of maintaining larvae-infected soil in celluloid receptacles under laboratory conditions. The cover to the large glass dish is not shown.

gram of dry soil was placed in these containers which produced a layer of soil about ½ in. deep. Fourteen cubic centimeters of water were added to this soil, followed by 1 cc. of water containing approximately 6,000 infective kidney worm larvae; these larvae were recovered in from 7- to 10-day-old cultures. The total amount of water added to the soil gave it a moist, but not a wet, consistency. After the larvae were added to the soil, the polyborate in powder or solution was either spread or sprayed evenly on the surface of the soil at the rate of 5 lb. (2,267.5 Gm.) to 100 sq. ft. (0.756 Gm. per receptacle dissolved in 3.8 cc. of water). When this chemical was applied in solution, it was dissolved at the rate of 5 lb. to 3 gal. of water. For each of the receptacles prepared and treated, a corresponding one was prepared and left untreated to serve as a control. After the soils were prepared, the receptacles were placed in large evaporating dishes upon small Stender dishes, which served as stands (fig. 2). In order to maintain the moisture of the soil throughout the experiment, a thin layer of water was kept at the bottom of each of the evaporating dishes; also, a few drops of water were added to the soils as needed. The evaporating dishes were kept covered, except for a small open area which permitted proper aëriation. The celluloid receptacles were kept in the above fashion for periods of from ten to twenty days, after which the larvae were recovered from the soils by the Baermann method. This consists of the use of a large clamped funnel containing warm (40 C.) water and fitted with a metal screen of fine mesh. At the end of four hours, 30 cc. of the water were withdrawn into a graduated cylinder and allowed to settle. Later, the top 15 cc. of water were siphoned out, and the remaining water was used in determining the number of larvae, if any, present. This was computed from the average of three counts of viable larvae present in a 1-cc. sample each of the mixed fluid. In making counts, the 1 cc. of fluid was spread in long and narrow streaks on a clean glass slide, approximately 3.5 by 7 in. in size and examined with a wide-field binocular microscope. Owing to the fact that some of these larvae were occasionally quiescent, it was necessary at times to touch them with a fine dissecting needle to test their viability.

In the outdoor observations, experiments were conducted either in an area adjacent to a building which was constantly shaded and protected from rain, or in a cool area next to shrubbery which received sunlight part of the day and was exposed to rainfall. These areas were chosen because, as observed by Spindler,[12] kidney worm larvae in hog lots are usually found in moist and shaded soil where they are protected from light and desiccation. In the areas which were chosen, the surface ground was thoroughly raked and all small rocks removed. The ground was then marked off by wooden borders in 1-square-foot plots.

In planning a suitable method for the concentration and recovery of the larvae from the soil, the following procedure was devised. Two small galvanized wire baskets (8-mesh to the inch), each 2¼ in. wide and ¾ in. high, were worked into the 1-square-foot soil plots. The soil within each basket was level with the ground and a counted number of larvae were then mixed within each confined area. Inasmuch as the larvae do not migrate extensively,[12] it was possible in this way, at the end of the experiment, to examine the soil within each basket and determine the condition of an adequate number of larvae. Following the addition

of the larvae in the soil as described, the entire surface soil within the plot was treated with polyborate. The amount used, either in dry form or in solution, was computed at the rate of 5 lb. per 100 sq. ft. (22.68 Gm. per sq. ft.). When the polyborate was applied in a dry form, the soil was lightly sprinkled with water just before and immediately following addition of the chemical. The soils which were kept as controls were left untreated. All these soils were examined after ten and twenty days. In the meantime, in order to maintain some moisture in the soil plots located in areas protected from rain, the soil was sprayed lightly with water once a day unless the plots received natural rainfall. At the end of the desired time, each wire basket was pulled out and the entire soil examined for larvae by the Baermann method. The number of larvae recovered were counted as described above except that, in most cases, concentrated HCl was added to the larval solution in dilution of 1:100 to destroy the free-living nematodes present. This technique, worked out by Shorb,[13] is a method which quickly destroys free-living nematodes and does not readily harm infective parasitic larvae. The acid was not used when the infective larvae recovered from the soils were intended to be fed to experimental animals; instead, the free-living nematodes, which as a rule were very few in number, were differentiated from the parasitic larvae by their general body size and motility.

In addition to the above observations, animal feeding experiments were conducted to obtain further evidence of the lethal action of polyborate on kidney worm larvae. Several young rabbits and 2 young pigs were fed treated and untreated soils or the larvae recovered from these soils. The soil was fed to the rabbits with the aid of a glass tube fitted with a glass piston; the soil was fed to the pigs by mixing it with dry feed.

Experimental Data and Results

Kidney Worm Larvae Recovered in Polyborate-Treated and Untreated Soils.—At the beginning of this investigation, about 6,000 infective kidney worm larvae were placed in soil within each of several celluloid receptacles. Some of the soils were treated; others were left untreated and served as controls. Following these preparations, the soils were examined at intervals of ten and twenty days. The results of these experiments, carried out three different times for each time interval, are summarized in table 1, experiments 1a and 3b.

Further tests were made outdoors under natural environmental conditions. Approximately 10,000 infective larvae were mixed with soil in each of two wire baskets within a 1-square-foot soil plot. When polyborate was applied, the entire area of each plot was treated. Plots containing corresponding infected soil in baskets and serving as controls were left untreated. For each experiment which was carried out at three different times, the soil in one basket was examined after ten days and the soil in the second basket after twenty days. The summary of experiments carried out in areas shaded continually and protected from rain, and in areas shaded only a part of the day and exposed to rain, are given in experiments 4a to 6b and experiments 7a to 9b, respectively, of table 1.

The results show that in tests carried out indoors, viable larvae in the treated soils, in contrast with the controls, ranged from 0.0 to 0.2 per cent (ave., 0.1 %) after ten days to 0.0 after twenty days. In experiments made outdoors where the larvae were maintained in completely shaded soils, live larvae in the treated soils ranged from 5.8 to 14.8 per cent (ave., 9.7 %) after ten days and 0.0 to 0.7 per cent (ave., 0.2 %) after twenty days. In outdoor experiments where the soils were maintained in shade part of the time and exposed to rainfall, larvae recovered from the treated soil ranged from 10.5 to 21.6 per cent (ave., 15.1 %) after ten days, and 0.0 to 0.3 per cent (ave., 0.1 %) after 20 days. A comparison of the number of larvae recovered in the indoor and outdoor experiments shows that more larvae were destroyed in the small receptacles during the first ten days of treatment than in the soils outside. This result undoubtedly was brought about by the heavier concentration of the chemical in the soil within the small receptacles. Twenty days after the polyborate treatment, all, or nearly all, the larvae were destroyed by the various methods used.

In experiment 10 (table 1), the larvae were mixed throughout the top 1 inch of soil within each of two 1-square-foot plots. The soil of one was treated and the other was left untreated. Twenty days later no larvae were recovered from a 50-Gm. sample of the treated soil, whereas 240 larvae were recovered from a similar quantity of untreated soil. The absence of live larvae in the treated soil was further confirmed by animal feeding experiments reported below (table 2, animal 2a).

Animal Feeding Experiments with Infective Larvae Exposed to Polyborate-Treated and Untreated Soils.—In addition to the experiments already reported, further tests were conducted through animal feeding on the viability of the kidney worm larvae exposed to treated and untreated soils. In this case, the animals were fed either the larvae-infected soil or larvae which had been recovered from these soils as recorded in table 1. Two-month-old rabbits were used for most of these tests, except in 1 case where 2, 4-month-old pigs were utilized. Rabbits were used

because they were highly susceptible to kidney worms and because it was possible to feed them moderate amounts (35 Gm.) of soil. The soil was usually fed in two feedings within a few hours of each other. When the pigs were used, the soil was mixed with dry feed and the total amount (3 lb.) was fed within two days.

The summary and results of these feeding experiments are given in table 2. This table shows that all rabbits (1, 2, 5, 6, 9, 11, and 13) which were fed untreated (control) soils, or the larvae recovered from these soils, displayed extensive worm damage of the liver one month after infection (fig. 3, B and D). The length of the kidney worms which were recovered at this time was approximately 2 to 5 mm. Four rabbits (3, 4, 7, and 8), which were fed soil from the celluloid receptacles, ten and twenty days after treatment with polyborate showed no liver lesions at necropsy. On the other hand, a few liver lesions were found in 2 rabbits (10, and 12, fig. 3, A) which were fed larvae recovered from outdoor soil plots ten days after treatment. No lesions were seen in the liver of rabbit 14 (fig. 3, C) which was fed a few larvae (only 5 estimated) recovered from soil twenty days after treatment. No kidney worm lesions were found in the liver of pig 2a fed 3 lb. of soil which had been removed from the entire surface of a 1-square-foot plot twenty days after treatment. A control pig (1a), however, which was fed a similar amount of soil from an untreated plot developed extensive lesions in the liver (fig. 4).

Observations in the Development of Kidney Worm Eggs and Preinfective Larvae in Polyborate-Treated Soil.—For these observations, two types of experiments were conducted and use was made of a large number of eggs recovered from freshly chopped adult female kidney worms. In one experiment, carried out indoors, the eggs were mixed with 35 Gm. of sterilized soil which had just been sprayed with the usual percentage of polyborate solution. This soil was kept in a celluloid receptacle 2.19 inches square. A corresponding, but untreated, soil was also prepared and

TABLE 1.—Number of Viable Infective Kidney Worm Larvae Recovered from Soils at Various Intervals Following Treatment with Polyborate

Exp. No.	Method used: (a) Chemical spread dry (b) Chemical sprayed		Approx. No. of larvae placed on soil	Duration of treatment (days)	Estimated number of viable larvae recovered		Viable larvae* (%)
					treated soil	untreated soil	
1a	Indoors: soil in celluloid receptacle	(a)	6,000	10	6	2,855	0.2
1b	Indoors	(a)	6,000	20	0	1,950	0.0
2a	Indoors	(a)	6,000	10	0	2,968	0.0
2b	Indoors	(a)	6,000	20	0	2,160	0.0
3a	Indoors	(b)	6,000	10	5	3,300	0.2
3b	Indoors	(b)	6,000	20	0	1,875	0.0
4a	Outdoors, natural conditions: larvae in soil within wire basket; soil shaded continually and protected from rain	(a)	10,000	10	585	3,959	14.8
4b	Outdoors	(a)	10,000	20	15	2,250	0.7
5a	Outdoors	(a)	10,000	10	154	2,634	5.8
5b	Outdoors	(a)	10,000	20	0	2,614	0.0
6a	Outdoors	(b)	10,000	10	222	2,565	8.7
6b	Outdoors	(b)	10,000	20	0	2,040	0.0
7a	Outdoors, natural conditions: larvae in soil within wire basket; soil shaded most of the day and exposed to rainfall	(a)	10,000	10	285	2,179	13.1
7b	Outdoors	(a)	10,000	20	5	1,995	0.3
8a	Outdoors	(a)	10,000	10	600	2,775	21.6
8b	Outdoors	(a)	10,000	20	0	2,895	0.0
9a	Outdoors	(b)	10,000	10	255	2,430	10.5
9b	Outdoors	(b)	10,000	20	0	2,085	0.0
10	Outdoors, natural conditions: larvae in soil spread evenly within 1-square-foot plot; soil shaded continually and protected from rainfall	(a)	15,000	20	0**	240**	0.0

* Number of larvae recovered from treated soil divided by number of larvae recovered from untreated soil; ** number of larvae recovered from a 50-Gm. soil sample.

TABLE 2—Results of Animal Feeding Experiments with Polyborate Treated and Untreated Soils Infected with Kidney Worm Larvae, or the Feeding of These Larvae Recovered from These Soils—The Animals Were Killed One Month Following Experimental Feeding

Animal No.	Type of animal used	Substance fed to animal	Soil treated or untreated	(a)—Approx. No. of larvae originally placed in soil (b)—Estimated No. of larvae fed	Duration of soil experiment (days)	Liver findings at necropsy 0—Liver normal; no lesions or kidney worms present. *—Liver not enlarged; few superficial larval tracts and small internal fibrotic areas containing young kidney worms. **—Liver enlarged; numerous superficial tracts and fibrous parenchyma containing young kidney worms.
1	Rabbit	35 Gm. of larvae-infected soil kept in celluloid receptacles	Untreated	(a) 5,000 (b) Undetermined	10	**
2	Rabbit	35 Gm. of larvae-infected soil kept in celluloid receptacles	Untreated	(a) 5,000 (b) Undetermined	10	**
3	Rabbit	35 Gm. of larvae-infected soil kept in celluloid receptacles	Treated	(a) 5,000 (b) Undetermined	10	0
4	Rabbit	35 Gm. of larvae-infected soil kept in celluloid receptacles	Treated	(a) 5,000 (b) Undetermined	10	0
5	Rabbit	35 Gm. of larvae-infected soil kept in celluloid receptacles	Untreated	(a) 5,000 (b) Undetermined	20	**
6	Rabbit	35 Gm. of larvae-infected soil kept in celluloid receptacles	Untreated	(a) 5,000 (b) Undetermined	20	**
7	Rabbit	35 Gm. of larvae-infected soil kept in celluloid receptacles	Treated	(a) 5,000 (b) Undetermined	20	0
8	Rabbit	35 Gm. of larvae-infected soil kept in celluloid receptacles	Treated	(a) 5,000 (b) Undetermined	20	0
9	Rabbit	Larvae recovered from shaded soil of exper. 4a, table 1	Untreated	(a) 10,000 (b) 3,959	10	**
10	Rabbit	Larvae recovered from shaded soil, exper. 4a, table 1	Treated	(a) 10,000 (b) 585	10	*
11	Rabbit	Larvae recovered from partly shaded soil, exper. 7a, table 1	Untreated	(a) 10,000 (b) 2,179	10	**
12	Rabbit	Larvae recovered from partly shaded soil, exper. 7a, table 1	Treated	(a) 10,000 (b) 285	10	*
13	Rabbit	Larvae recovered from partly shaded soil, exper. 7b, table 1	Untreated	(a) 10,000 (b) 1,995	20	**
14	Rabbit	Larvae recovered from partly shaded soil, exper. 7b, table 1	Treated	(a) 10,000 (b) 5	20	0
1a	Pig	3 lb. of larvae-infected soil scraped from entire surface of soil plot of exper. 10, table 1, plus 240 larvae recovered from sample of soil	Untreated	(a) 15,000 (b) Undetermined	20	Surface rough with whitish discoloration and numerous internal fibrotic nodules containing young worms.
2a	Pig	3 lb. of larvae-infected soil scraped from entire surface of soil plot, exper. 10, table 1	Treated	(a) 15,000 (b) Undetermined	20	0

used as a control. In another experiment, outdoors, the eggs were mixed with a small, superficial portion of soil of a 1-square-foot soil plot freshly sprayed with polyborate solution. The eggs were also mixed with a corresponding, but untreated, soil and maintained as a control. Each day a small amount of water was sprayed on each of the above plots in order to keep the soil slightly moist. At the end of five days, each of the above soils was examined for larvae by the Baermann method. This yielded many viable infective larvae from the untreated soils, and only a few degenerating preinfective larvae from the treated soils; most of these degenerating larvae were dead, but a few showed feeble movement.

The above experiments, which were repeated with the same results, show that in treated soil some of the eggs developed and hatched normally, but the preinfective larvae were killed before attaining the infective stage.

In order to ascertain the residual effects of polyborate, a large number of freshly secured kidney worm eggs were mixed with approximately 25 Gm. of soil taken from the surface of an outdoor soil plot thirty-five days after it had been treated with dry polyborate. The soil sample was kept indoors in a Stender dish. A similar soil-egg mixture was also prepared with the use of untreated soil taken in the vicinity of the above treated soil and kept as control. At the end of five days, when these soils were examined by the Baermann method, a large number of infective

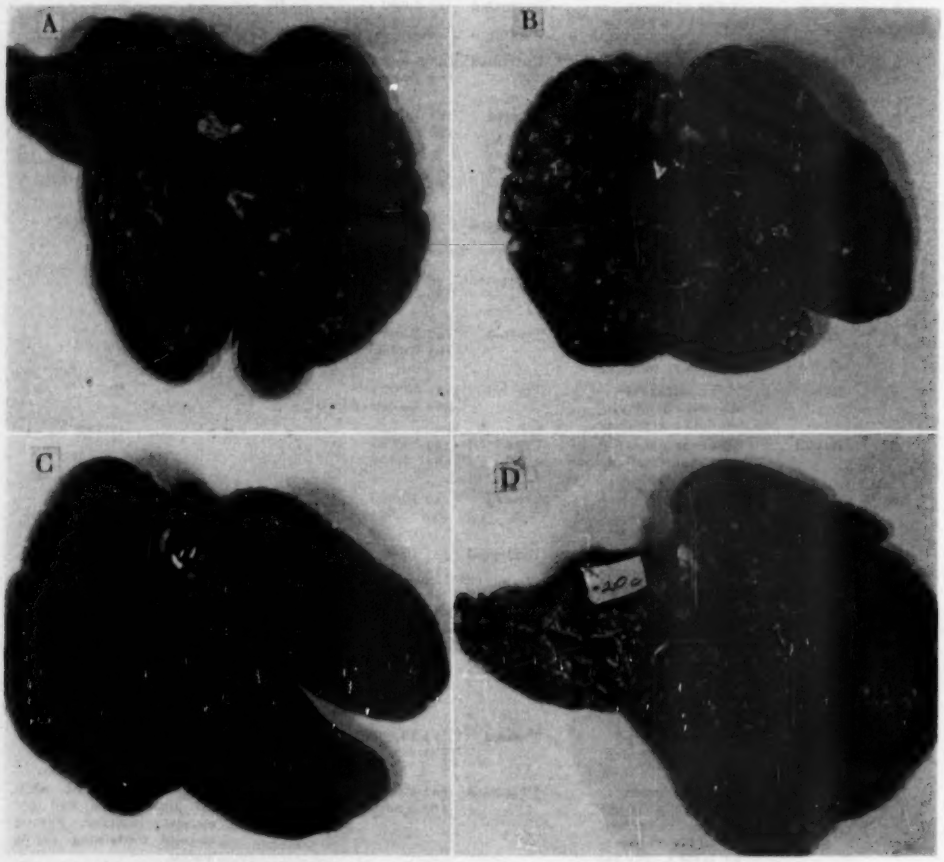

Fig. 3—(A) and (C) livers of rabbits (12 and 14, table 2) which had been fed kidney worm larvae recovered from soils ten and twenty days, respectively, after treatment with polyborate; (B) and (D) livers of corresponding control rabbits (11 and 13, table 2) which had been fed larvae recovered from untreated soils. Note extensive lesions in (B) and (D) in contrast with few lesions in (A) and none in (C).

Fig. 4—Livers of pigs slaughtered thirty days after the pigs were experimentally fed soil containing approximately 15,000 infective kidney worm larvae.

(Left) Liver of pig which was fed the infected soil twenty days after it had been treated with "polybor-3"; note normal color and absence of lesions.

(Right) Liver of pig which was fed the control untreated infected soil; note enlargement, discoloration, and extensive kidney worm lesions.

larvae were recovered from both treated and untreated soils. Mention should be made, however, that the treated soil had been subjected to heavy rainfalls following treatment, which may account for the dilution of the chemical in the soil.

Observations of the Toxic Effect of Polyborate on Swine.—Studies carried out by Hoerlein[8,9] have shown that dogs kept in kennels which had been treated with sodium borate "borascu" developed no toxic symptoms. He reported, however, that feeding of 1.5 Gm. of this product to young puppies, produced anemia and leukopenia. Owen[10] noted no adverse effects on the health of dairy cows as a result of prolonged borate feeding. Raynor and Britton[11] reported no ill effects among ewes which were fed an ounce of borax daily for fifteen days.

In order to ascertain any ill effects of polyborate on swine, a 4-month-old pig was enclosed for one month on a plot of ground approximately 13 by 11 ft. This ground had been treated with dry polyborate one day before the pig was brought in, and again two weeks later while the pig was in the enclosed area. The soil was sprayed with water just before and after application of the polyborate for the purpose of dissolving the chemical. At the beginning of this experiment, and one month later at the end, the weights and blood counts of the pig were as follows: (at beginning) weight, 71.5 lb.; red blood cells, 5,000,600; white blood cells, 17,600; hemoglobin, 11.6 Gm. per 100 cc.; (at end) weight, 112 lb.; red blood cells, 6,000,060; white blood cells, 16,500; hemoglobin, 11.6 Gm. per 100 cc. The above figures indicate that the pig gained weight normally (1.3 lb. per day) and the blood count remained within normal limits. No irritation of the skin was noted following daily spraying of the pig with 10 per cent polyborated solution for a period of seven days.

Discussion

Data is presented in this paper which shows that under the conditions of the experiments, polyborate was effective in destroying the vitality of most kidney worm infective larvae within approximately twenty days. In freshly treated soil, the eggs appeared to have hatched normally, but the preinfective larvae were killed before reaching the infective stage. Furthermore, when this chemical was used judiciously on soil it did not produce ill effects to a pig present in the treated area. The results of the experiments therefore warrant further observations on the practical use of polyborate for the control of kidney worm larvae in the soil in ordinary swine-raising practices.

Since polyborate is injurious to vegetation, it can be used only on bare ground or where injury to vegetation is of no importance.

Even though polyborate is relatively nontoxic, in order to reduce any possible danger to foraging animals, it appears best to apply it in solution rather than in dry form. If it is used in dry form, it should be spread on quite wet ground following rainfall. The best localities for treatment in hog lots are areas which are shaded and kept moist a great deal of the time, since, according to published reports, these are the localities where kidney worm larvae are most commonly found.

Although polyborate appears to have limited residual effect, no doubt dependent upon the type of soil and amount of rainfall, nevertheless, it can be utilized to good advantage in (a) rendering an already infected hog lot comparatively free of kidney worm larvae, and (b) in maintaining, by frequent application, a low level of kidney worm larvae in soil where infected animals are kept. The use of this form of soil treatment over an extended period of time, incorporating it with other forms of sanitary practices, should assist in bringing about an adequate control of kidney worms in swine.

A method for the application of polyborate on soil under field conditions has been suggested by the writer and published elsewhere.[14]

Summary

Polyborate, a mixture of sodium pentaborate tetrahydrate and sodium tetraborate pentahydrate, has been used under laboratory and simulated field conditions for the purpose of evaluating its efficacy in destroying the eggs and the free-living larval stages of the swine kidney worm, *Stephanurus dentatus*.

The soil was artificially infected by mixing a known number of infective kidney worm larvae with a layer of soil about ½ in. thick in small celluloid receptacles under laboratory conditions and by mixing these larvae with the top ¾ to 1 in. of soil in small plots under field conditions.

The chemical was applied dry or in solution on the surface of the infected soils at the rate of 5 lb. to 100 sq. ft.

The efficacy of the polyborate was determined by comparing, by the Baermann method, the number of viable larvae in the treated soil with those of the untreated

soil, ten and twenty days after treatment, and, also, by comparing the degree of liver injury produced in susceptible animals following the feeding of infected treated or untreated soils, or by feeding the larvae recovered from these soils.

In small outdoor soil plots in shaded and partly shaded areas, the percentage of viable larvae, in contrast with corresponding controls, averaged from 9.7 to 15.1 per cent ten days after treatment and 0.1 to 0.2 per cent twenty days after treatment.

Rabbits which were fed all the larvae recovered from soils ten days after treatment showed only a few lesions in the liver at necropsy one month later, and a rabbit fed all larvae recovered from soil twenty days after treatment showed no liver lesions. Control rabbits fed larvae from nontreated soils developed extensive liver lesions. Furthermore, a pig fed infected soil twenty days after treatment displayed no liver lesions at necropsy, whereas the corresponding control pig fed the untreated soil developed numerous liver lesions.

Where fresh, kidney worm eggs were mixed with soil recently sprayed with polyborate solution, no live infective larvae were recovered five days later, whereas a large number were recovered from the untreated control soils. In the treated soils, dead preinfective larvae were found, indicating that some of the eggs had hatched normally, but the larvae apparently were killed before attaining the infective stage.

When kidney worm eggs were placed in a soil sample thirty-five days after it had been treated many infective larvae were recovered five days later. This soil had been exposed to considerable rainfall following treatment.

A 4-month-old pig kept for one month in a small, outdoor, enclosed area twice treated with polyborate showed no ill effects from exposure to the treated soil.

It is concluded that polyborate is an effective agent for the control of kidney worm larvae in soil, and warrants further observations on its use under ordinary swine-raising practices. Polyborate is injurious to vegetation and should not be used where vegetation is to be maintained.

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Isolation Units for Growing Baby Pigs Without Colostrum

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IN A PREVIOUS paper [1] it was shown that, under the proper environmental conditions, baby pigs can be raised without colostrum. This finding was contrary to the earlier experiences of others [2] who had, for the most part, reported failure in attempts to raise pigs which had not received colostrum. More recently, however, Sheffy *et al.*[3] have demonstrated that baby pigs can be so raised when phospholipids and antibiotics are included in the diet. Antibiotics apparently control some of the infections which would otherwise kill the young pigs that have not had the benefit of colostrum.

Our purpose in growing young pigs without colostrum has been to secure a disease-free and antibody-devoid animal for virus transmission studies. Because individual isolation is essential in the study of highly transmissible diseases, we have devised an isolation unit suitable for housing individual pigs from birth to 4 weeks of age. The purpose of this paper is to describe these isolation units, and to indicate their usefulness in the field of swine research.

From The Hormel Institute, University of Minnesota, Austin.

Hormel Institute publication 84, and paper 2943, scientific journal series, Minnesota Agricultural Experiment Station.

Dr. Young is associate professor in veterinary medicine, and Mr. Underdahl is assistant scientist.

DESCRIPTION OF APPARATUS

The isolation equipment to be described is similar to that described by Horsfall and Bauer,[4] but the individual units have been adapted to the requirements for feeding and maintaining pigs. These requirements are somewhat different from those for most laboratory animals.

A view of the isolation units set up in a battery of six is given in figure 1. The units are exhausted through a 4-in. manifold by means of a No. 53

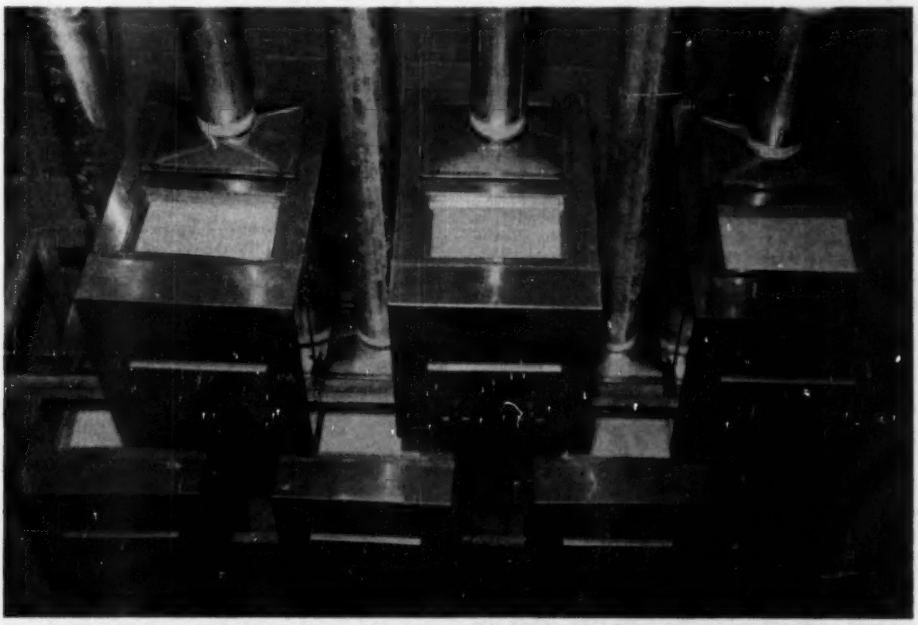

Fig. 1—Battery of six isolation units. Picture taken from front and above units. Pipes carry exhaust air from unit header to battery manifold.

junior blower* operated by a 0.04 HP 1,550 r.p.m. Universal motor. The blower, as we use it, provides approximately 10 air changes per unit per hour. A disassembled isolation unit is pictured in

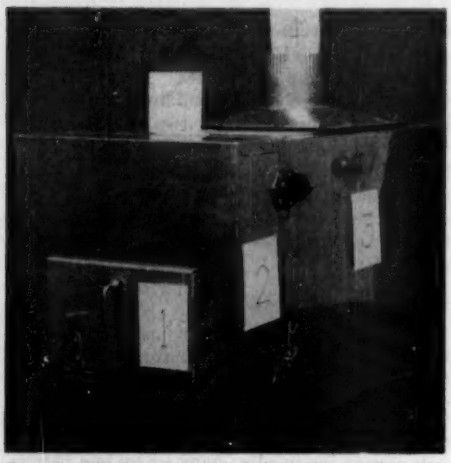

Fig. 2—Single assembled isolation unit: (1) feeding door; (2) service door; (3) unit proper; (4) air exhaust; (5) air intake.

figures 2, 3, 4, and 5, with accompanying identification numerals and description.

The principle on which the isolation unit works is that all air which enters or leaves the units must pass through the filters; hence, the unit is tightly sealed. The service door is provided with a rubber gasket, and is held tightly against the unit wall by means of four trunk hasps. The feeder door also has a gasket which is held tightly to the service door by a tension hatch. The exhaust header is provided with a gasket and is held tight by means of four wing nuts. The observation window, a 150-mm. pyrex watch glass, is sealed in place with plastic cement.

Filterdown pads,** No. 50-FG, are used to filter both intake and exhaust air. These pads come in sheets 24 by 36 in. and are easily cut with scissors. They are sterilized for use in the units by means of formaldehyde gas produced by adding 10 cc. of formalin to 5 Gm. of potassium permanganate.

EXPERIMENTAL

Baby pigs for the isolation units are obtained in one of two ways—either they are caught at birth in a sterile canvas bag, or they are removed during hysterectomy. In the latter case, since they do not traverse the birth canal, the pigs are less likely to have infections common to the dam and her environment. Immediately after birth, the navels are tied and dipped in iodine. All pigs are given 1 mg. of vitamin K either orally or by injection. The pigs are then placed in the units for eight to twelve hours before being fed. The temperature of the housing is kept between 90 and 95 F. the first twenty-four hours, and thereafter may be dropped to 85 F.

The diet currently used is basically homogenized cow's milk containing 6.5 per cent butterfat. The increased butterfat is obtained by mixing cereal cream with homogenized milk. To 1½ quarts of milk are added the yolk of one egg and 5 cc. of mixed inorganic salts.† No additional vitamin A is added since the amount supplied by the milk and egg yolk is adequate. Six to eight hours after birth, the pigs are offered enough milk to cover the bottom of the feeder pan. The pigs drink this milk without any additional inducements. Pigs caught by the sterile bag technique in the late afternoon or during the night may be fasted until the next morning. One quart of milk per feeding of 6 pigs is offered at 8 a.m., at noon, and at 4 p.m. the second day. No night feedings are necessary as the pigs are maintained well on three daytime feedings. The amount of milk may be increased gradually as the capacity of the pigs increases. In baby pigs fed as described above, diarrhea is rare. When it occurs, it is mild and usually lasts less than one day. Overfeeding is the most frequent cause.

Proper handling of the feeder pans is very important to the successful operation of the isolation units. Two sets of pans are desirable so that one set may be left in place in the units. With normal pigs, the pans are first rinsed with tap water to remove the milk, and then soaked in an antiseptic bath. They are rinsed again with tap water just before use. With animals being used for disease transmission, the feeder pans are autoclaved. The feeding technique is simple enough so that the

* New York Blower Co., LaPorte, Ind.

** American Filter Co., Louisville, Ky.

† The salt mixture consisted of 49.8 Gm. of $FeSO_4 \cdot 7H_2O$, 3.9 Gm. of $CuSO_4 \cdot 5H_2O$, 3.6 Gm. of $MnCl_2 \cdot 4H_2O$, 0.26 Gm. of KI, and water to make 1 liter. This will form a precipitate; resuspend before using.

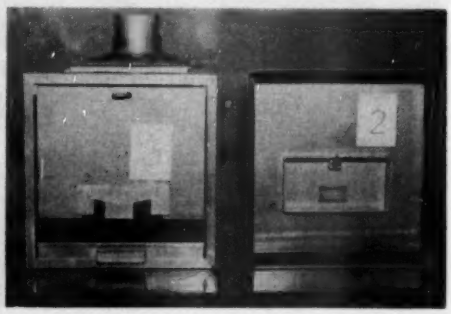

Fig. 3—Service door (2) has been removed from unit exposing false front (3). Feeder tray is in place below numeral 3.

average animal attendant, after a short training period, can feed and care for both normal and infected pigs at the same time.

The units described above have been in operation since February, 1952, and have proved very useful for virus transmission studies. Up to submission of this manuscript, 161 pigs have lived in the units, 56 collected by the bag technique and 105 by hysterectomy. Nineteen (11.8 %) of the pigs have been lost but of these, 11 were apparently lost because of a feeding error. A pint of week-old cream was mixed into the diet and fed before it was noticed to be sour. Disregarding these 11 pigs, only 8 (5 %) of 150 pigs were lost, which is only slightly higher than might be expected from developmental anomalies and incidental losses.

DISCUSSION

The isolation units here discussed have been subjected to an unusually stringent test of their ability to protect individual pigs. After five days, pigs housed in them have been inoculated with transmissible gastroenteritis virus (tge), an agent that transmits readily on inhalation or by contact with contaminated objects. Pigs so inoculated have been used to determine quantitatively the amount of infectious agent in various tissues. In such a titration, it is desirable to have an extinction end point as indicated by the inability of higher dilutions to cause infection. Typically, 1 or more pigs become infected and are violently ill while other pigs, in the same titration but given more dilute material, remain normal.

Although the principal use of the isolation units has been to study viral diseases in swine, their usefulness is not limited to a study of swine disease. There is a good probability that there are diseases of man and swine which are interchangeable. Baby pigs that have not received colostrum should make excellent hosts for experimental transmission of such human disease agents, whereas pigs which have nursed their dams might be poor hosts because of the immunological background of the dam. Such antibody as appears in baby pigs is transferred via the colostrum, so that young pigs which have had colostrum might not show true susceptibility of pigs to human infectious agents because of previous experience of their dam with that agent. From this point of view, it would seem well worthwhile to investigate the usefulness of disease-free, antibody-devoid baby pigs as possible experimental animals for those human diseases for which no satisfactory experimental host has been found.

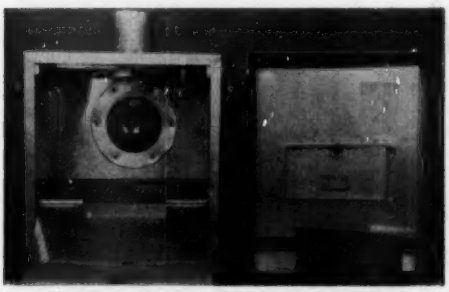

Fig. 4—View similar to figure 3. Feeder tray has been removed and false front has been dropped down. Observation window and false bottom are apparent.

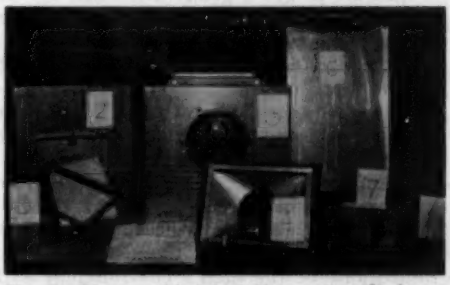

Fig. 5—Disassembled isolation unit: (2) inner surface of service door; (3) back of unit proper; (4) unit header and filter; (5) air inlet filter and holder; (6) waste tray; (7) feeder tray; and (8) false bottom.

Another use of the isolation units and baby pigs which have not had colostrum would be to study the basic nutritional requirements of the baby pig. For the most part, colostrum has been considered essential in the diet primarily for nutritional reasons. The evidence presented here and by Sheffy *et al.*[3] suggests that colostrum is essential only from the standpoint that immune lactoglobulins protect the pig against the diseases common to his natural environment. The diet described in this paper is satisfactory for our experimental purposes, but it is far from ideal, since the gains made by pigs during the first two weeks on the diet are not as good as those of "good doers" with the sow. Thus, exploration of the nutritional requirements of the baby

pig in a disease-free environment could result in fundamental information which might be very useful.

Under present management systems, it is difficult to get a good expression of the genetic growth capacity of the suckling pig. During the first few weeks, the growth is largely, if not entirely, an expression of the capacity of the dam to transmit protective antibodies and to provide sufficient milk. Thus, the performance of a litter during the first few weeks is a measurement of sow performance. With moderate improvement in the diet now used, the isolation units could provide a measure of true growth capacity in the absence of the inhibitions of the dam's environment and her lactational limitations. Correlation of such early growth data with postweaning data could conceivably make possible a better selection of animals for the growth qualities desired.

A fourth important potential use of the isolation units is for ridding valuable blood lines of diseases such as atrophic rhinitis, tge, brucellosis, dysentery, and erysipelas. Pigs taken from good gilts and raised on milk in isolation units could later be adapted to ordinary farm conditions, thus creating a break in the pig-to-pig contact essential to the transmission of many swine diseases. A small colony of swine has been so established, and the pigs have reached breeding age free of the two diseases (tge and dysentery) which plague the herd from which they were taken. Adaptation of such pigs to ordinary environmental conditions is not as difficult as might be expected because maturation alone cushions the transition from laboratory to farm environment.

SUMMARY

Isolation units are described which were used to raise pigs from birth to 4 weeks of age. Because a disease-free environment was provided, colostrum was not essential. The usefulness of these units for the study of (1) disease, (2) nutritional requirements of baby pigs, (3) genetic capacity for growth, and (4) disease control is discussed.

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Observations on an Erythrocytic Inclusion in Swine

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DURING the course of field investigations on Eperythrozoon infections in swine, an erythrocytic inclusion of unusual character was noted in the blood of several pigs in a herd under observation. A study made of these intracorpuscular structures has revealed them to be iron-reacting bodies similar in some respects to the inclusions noted by Pappenheimer *et al.*[1] and others [2-4] in cases of human anemias of obscure etiology. Although the significance of the swine inclusions has not been determined, the observations made in connection with the study of these structures may be of interest.

LITERATURE CITED

The presence of iron-reacting erythrocytic inclusions in human subjects has been reported in recent years by several investigators. In the majority of cases, inclusions appeared following splenectomy, which was used in each case as a therapeutic measure for various types of anemia present.

Pappenheimer, *et al.*[1] attempted to identify these structures by culture, transmission studies, and other methods but no definite conclusions were reached in regard to their etiology. MacFadzean and Davis[2] concluded the inclusions were an expression of faulty iron metabolism. They and others[3, 4] have found similar bodies following treatment by splenectomy of familial anemias due apparently to hereditary factors.

The various types of iron-reacting erythrocytic inclusions have been classified by Mills and Lucia.[4] They consider the inclusions of Pappenheimer *et al.*[1] to be an expression of faulty iron metabolism due to toxins, and the structures associated with familial anemias[2-4] to be due to a congenital defect in iron metabolism.

CLINICAL DATA

The herd under investigation consisted of 32 Poland China pigs averaging approximately 80 lb. in weight. At the initial visit, 1 pig was dead and 3 others were sick. A diagnosis of acute infection with *Eperythrozoon suis* was made following clinical observations and microscopic examination of blood films from the affected animals. Blood films also were prepared at this time from 15 apparently normal pigs selected at random from the herd.

In 2 of the sick pigs (1 and 2) intraerythrocytic bodies (described below) were observed in approximately 20 per cent of the erythrocytes, together with numerous forms of *E. suis*. Eperythrozoa, only, were present in the 2 other affected animals. Identical inclusions unassociated with eperythrozoa were found in approximately 2 per cent of the red blood cells of 2 apparently normal pigs (3 and 4). Mild Eperythrozoon infections, only, were present in 4 other animals of the latter group.

In addition to the usual clinical symptoms of acute eperythrozoönosis, pigs 1 and 2 displayed a hemoglobinuria. Pigs 3 and 4 remained normal clinically during the two-month period in which the herd was under observation. There was no significant change in the frequency of the inclusions in these 2 animals.

One of the acutely affected pigs (1) was placed under observation in the laboratory. Sixty days transpired before blood values returned to normal. This slow rate of improvement was attributed in part to the persistent hemoglobinuria which occurred intermittently during this period. Intraerythrocytic inclusions remained numerous at all times, but gradually decreased in frequency as the polychromatic erythrocytes reduced in numbers.

MORPHOLOGY AND STAINING

The majority of inclusions occurred within the polychromatic erythrocytes and, occasionally, in mature red cells. Their frequency was usually directly related to the number of immature erythrocytes present in the blood. Inclusions also were observed frequently in the cytoplasmic portion of normoblasts, and occasionally in that of erythroblasts. The bodies took a reddish purple or purplish blue color with Giemsa's or Wright's stain.

The predominating structure (fig. 1) appeared coccoid, averaging about 0.8 μ in diameter. Erythrocytes contained from one to as many as 16 or more of these inclusions. Diploid and tetrad forms were often seen, and clusters of five to ten cocci within individual erythrocytes were numerous. Some were seen to possess a small filamentous attachment.

There were also present (fig. 2) round or ovoid forms consisting of an outer ring of reddish purple material surrounding a large vacuolated central area. These inclusions varied from 0.7 to 4.7 μ in diameter and averaged about 2.5 μ in size. The number of these forms varied from one to as many as eight in individual erythrocytes. They were observed most readily toward the end of blood films at which point the erythrocytes were flattened during the smearing technique.

Under supravital staining, the inclusions could be observed to exhibit Brownian movement and to

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From the Kansas Agricultural Experiment Station as a collaborator under the North Central Regional Agricultural Experiment Station coöperative research project entitled "Death Losses in Young Pigs."

change their relative positions within the erythrocytes, but no definite motility could be detected. In blood films treated with hydrochloric acid (3 parts of a 1 % solution) and potassium ferrocyanide (1 part of a 2 % solution) the bodies gave a positive iron reaction (Prussian blue reaction). They did not retain Gram's stain. Attempted culture of blood on the usual laboratory medium yielded negative results.

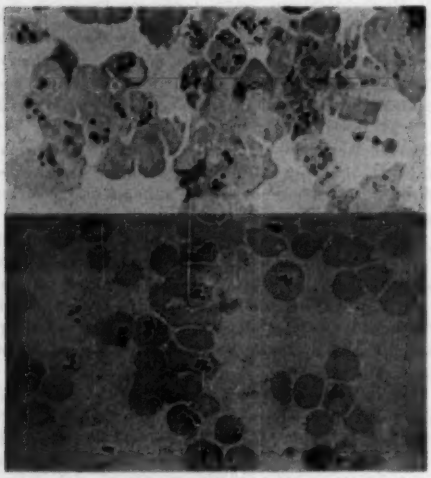

Fig. 1—Coccoid erythrocytic inclusions.

EXPERIMENTAL

The unusual appearance of these inclusions and their occurrence in a number of animals in a single herd suggested the possibility of their being a form of benign hemotropic parasite.

Experimental transmission to other swine was attempted using whole blood (defibrinated, citrated, or immediate injection without alteration) from pigs 1, 3, and 4. Splenectomized and unoperated pigs received subcutaneous, intramuscular, or intravenous injections but failed to develop erythrocytic inclusions following observation periods of from one month up to a year. Transmission also failed using 3 cc. of bone marrow fluid obtained from pig 1, and injected into the sternal marrow of a splenectomized pig.

Splenectomy (pig 1) did not appreciably increase the numbers of inclusions. However, their frequency did increase during the periods of anemia produced by relapses of *E. suis* following splenectomy. The increase in inclusions appeared to be due to the greater numbers of immature erythrocytes present. Intravenous doses of neoarsphenamine injected into pig 1 were effective in clearing the blood of eperythrozoa but had no effect in reducing the numbers of erythrocytic inclusions. Attempted transmission using a suspension of the spleen obtained from pig 1 at splenectomy also resulted in failure.

Because of similarities in the appearance of the structures to Anaplasma, a splenectomized calf was injected intravenously with citrated blood from pig 1. No erythrocytic inclusions developed in the calf; after ninety days elapsed, it was injected with Anaplasma carrier blood and proved susceptible to acute anaplasmosis.

DISCUSSION

The transmission failures suggest the probability that the inclusions are not directly related to any infectious agent. In addition, none of the erythrocytic parasites are known to give a positive reaction to the Prussian blue test for iron.[1] Although a close resemblance to nuclear remnants is suggested, this possibility seems to be eliminated by the iron reaction of the inclusions and their occurrence in the cytoplasm of normoblasts and erythroblasts with nuclei intact.

The intraerythrocytic bodies bear a strong resemblance to those reported in human subjects in their morphology, their presence in cells of the erythrocytic series, their positive reaction to iron, and their occurrence in closely related individuals. In a comparison of the morphology with human inclusions, Dr. Alwin M. Pappenheimer * has written as follows:

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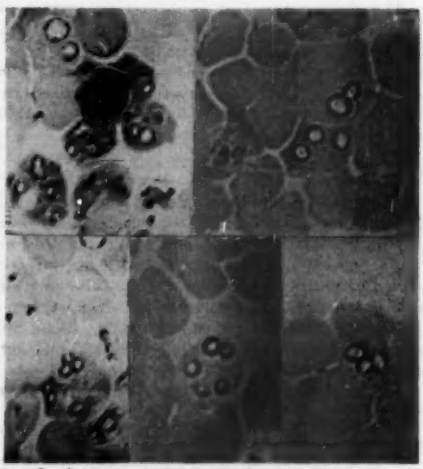

Fig. 2—Large ovoid inclusions with centrally located vacuoles.

I am sending you a slide of one of our patients with febrile anemia. As you will see, the erythrocytes contain bodies which seem to be not identical, but at least of the same character as those you have found in your swine. I have the impression that they are perhaps slightly smaller.

Marked differences between the inclusions of swine and human beings include the appearance of the swine bodies in large numbers prior to splenectomy, the apparent ineffect of splenectomy on their numbers, and the absence of any definite pathology attributable to the presence of the inclusions. While the hemolytic anemia observed in 2 of the pigs may possibly have been directly associated with the presence of the inclusions, this does not appear likely in view of the large numbers of Eperythrozoon present and the acute onset of the anemia. This rapid onset is in contrast to the usually chronic progressive anemia of human subjects found associated with erythrocytic inclusions. The significance of the hemoglobinuria noted in 2 of the pigs is not clear. This symptom is not characteristic of either acute eperythrozoönosis or the anemias of man associated with inclusions.

Although no definite conclusions can be reached in regard to the significance of the structures observed, it appears probable that they arise from factors related to those which cause the appearance of similar erythrocytic bodies in human subjects. If this is true the possibility is suggested that a chronic progressive anemia may occur occasionally in swine similar to the anemias associated with erythrocytic inclusions in man.

SUMMARY

Four pigs in a single herd were found to be harboring iron-reacting inclusions in cells of the erythrocytic series of the blood. The inclusions were associated with an acute hemolytic anemia attributed to Eperythrozoon infection in 2 of the animals, and apparently unassociated with any pathology in 2 others. The similarity of these structures to those observed in human patients affected with congenital familial anemia and acquired hemolytic anemias of unidentified etiology is discussed.

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Differentiation of the Ova of *Ancylostoma Caninum* and *Uncinaria Stenocephala* in Dogs

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IN EVALUATING dogs for anthelmintic drug testing, it was observed that two types of hookworm ova appeared in routine fecal flotations. One type of ovum was small and was found frequently; the other type was larger and was found infrequently.

In attempting to identify these ova, several sources of literature were consulted including original descriptions. The range of size variations given by different authors (table 1), however, left some doubt in the writer's mind as to which source or sources were correct.

In order to resolve the problem, collections of several hundred adult hookworms at autopsy revealed that *Ancylostoma caninum* and *Uncinaria stenocephala* were the only Ancylostoma spp. found in a series of 147 dogs. The measurements of ova presented in this paper were obtained from this series of dogs. These dogs were examined during the latter six months of 1952 and were obtained from the states of Indiana, Illinois, Kentucky, Michigan, Ohio, and Tennessee. During this period *U. stenocephala* had been observed by the writer in dogs from Ohio, Illinois, Indiana, and Kentucky. Further data regarding the incidence of *A. caninum* and *U. stenocephala* will be published.

Examination of ova, from *A. caninum* and *U. stenocephala* teased out of adult female hookworms in physiological saline, indicated clearly that *A. caninum* produced the smaller ovum while *U. stenocephala* produced the larger ovum (table 2 and fig. 1). The range of sizes of the ova of these two species in several mediums are shown in table 2.

DISCUSSION

The literature contains radical discrepancies concerning the sizes of ova of *A. caninum* and *U. stenocephala*. These inconsistencies are most probably due to: variations resulting from the use of different mediums, failure to see the proper ova, failure to differentiate species of hookworm ova, typographical errors in transposition, and the perpetuation of erroneous data without observation.

The data in table 1 represent ova sizes for *A. caninum* and *U. stenocephala* as given by the authors indicated. When compared with the data obtained in table 2, only six of the 11 values given for *A. caninum* approach the correct range. Only three of the seven values given for *U. stenocephala* approach the true range. Also, examination of the six dual citations on *A. caninum* and *U. stenocephala* shows that only one is correct.

The data in table 2 show ova sizes of *A. caninum* and *U. stenocephala* as obtained in several mediums. These data indicate beyond doubt that *U. stenocephala* produces a larger ovum than does *A. caninum*. Using minimum and maximum figures obtained, the range for *A. caninum* is 55-74 x 37-43, and the range of *U. stenocephala* is 71-93 x 37-55. The figures obtained agree closely with the values given by Sarles,[20] Monnig,[14] Landsberg,[13] Monnig,[15] and

TABLE 1—Measurements of Ova in Microns as given by Various Authors

Authors	*Ancylostoma caninum*	*Uncinaria stenocephala*
Railliet (1884)	*	63-76 x 32-38 (*in utero*)
Hall (1923)	74-84 x 48-54	63-67 x 32-38
Fulleborn (1924)	*	*
Ransom (1924)	*	*
Hall (1925)	*	*
Yorke and Maplestone (1926)	*	*
Sarles (1929)	62.17 x 37.87 (mean)	*
Baylis (1929)	*	*
Sprehn (1932)	74-84 x 48-54	85 x 49 (by using scale)
Hall *et al.* (1934)	*	*
Neveu-Lemaire (1936)	74-84 x 48-54	63-76 x 32-38
Monnig (1938)	56-65 x 37-43	82 x 47
Landsberg (1939)	63.8 x 40.4	*
Belding (1942)	60-75 x 38-45	*
Monnig (1947)	56-65 x 37-43	65-80 x 40-50
Koutz and Rebrassier (1948)	*	*
Benbrook and Sloss (1948)	Photo without size reference	*
Morgan and Hawkins (1949)	74-84 x 48-54	*
Koutz (1949)	*	*
Craig and Faust (1951)	63.8 x 40.4 (average)	*
Univ. Ill. Circ. 698 (1952)	Photos of each showing no discernable size or shape differences	
Coffin (1953)	80 x 50	65 x 40

* Either no ova size given or not mentioned.

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Craig and Faust.[5] There is no significant overlap of size ranges of the ova of these two species. An occasional *A. caninum* ovum will be distorted to the length of an ovum of *U. stenocephala*, but the diameter still falls within the typical range for *A. caninum*.

These data reflect to some extent distortion due to the mediums used.

In figure 1 may be seen the essential differences between typical ova of *A. caninum* and *U. stenocephala*. Slight distortion of the inner membrane can be seen in both species. This sort of distortion occurs in all the flotation mediums tested.

It is significant that *U. stenocephala* was mentioned only once in a series of 17 publications dealing with canine helminth parasites in the United States from 1922 to 1952 (in addition to the citations given in table 1). The paucity of information regarding Uncinaria seems to indicate an oversight in observing adults and ova because of faulty technique, or failure to recognize the species as adults or ova. It is more than likely that the distribution of *U. stenocephala* exceeds greatly that of Alaska and Maryland as Dikmans[6] indicates.

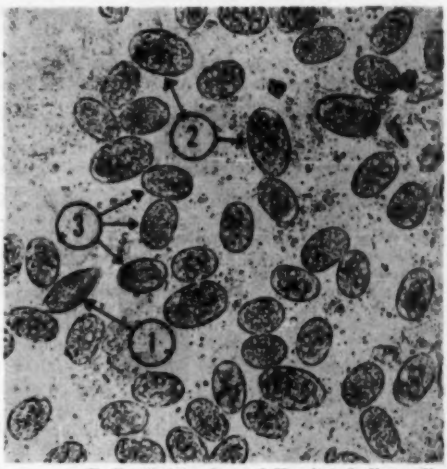
The data presented should provide an accurate and readily available means of recognizing and distinguishing between the ova of *A. caninum* and *U. stenocephala*, and thus indirectly result in a greater clarification of the distribution and incidence of these two species, in particular that of *U. stenocephala*.

This is another case of small worms (Uncinaria) producing larger ova, while large worms (Ancylostoma) produce smaller ova. The same situation is found in equine parasites where small strongyles (Triodontophorus, Craterostomum, etc.) produce larger ova and large strongyles (Strongylus) produce smaller ova.

TABLE 2—Sizes of Ova from Ancylostoma Caninum and Uncinaria Stenocephala in Various Mediums*

Medium	*Ancylostoma caninum*	*Uncinaria stenocephala*
Saline, *ex utero*	62 x 37-43	80-86 x -49
Lactophenol, *in utero*	62-68 x 37	74-83 x 46-55
Sodium dichromate direct flotation	55-74 x 37	74-86 x 39-49
Sodium dichromate centrifuged flotation	58-68 x 37	74-93 x 43-49
Zinc sulfate direct flotation	55-62 x 37-43	74-93 x 43-49
Zinc sulfate centrifuged flotation	55-74 x 37-43	74-80 x 43-49
Sodium nitrate direct flotation	55-68 x 37	74-86 x 43-49
Sodium nitrate centrifuged flotation	62-68 x 37-43	93 x 37-43
Sugar direct flotation	62 x 37	71-80 x 43-49
Sodium chloride direct flotation	62-65 x 37-40	74-80 x 43

* Size ranges represent measurements on 10 ova observed from several dogs.



—R. F. Albright, General Hospital, Indianapolis.

Fig. 1—Photomicrograph of (1) Trichuris ovum, (2) Uncinaria ova, and (3) Ancylostoma ova.

SUMMARY

1) The literature contains discrepancies in the information regarding the ova sizes of *Ancylostoma caninum* and *Uncinaria stenocephala* in dogs.

2) Data are presented on the sizes of ova in several mediums, and a photomicrograph of typical ova clearly shows that *A. caninum* produces ova smaller than those of *U. stenocephala*.

3) The differentiation of these two ova provides a means for clarifying the incidence and distribution of *U. stenocephala*.

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The English Sparrow as a Natural Carrier of Newcastle Disease Virus

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FREE-FLYING birds are suspected of being involved in the spread of Newcastle disease (ND) as infected carriers. The English sparrow *(Passer domesticus)* was chosen as the subject for these studies because of its large population, wide distribution, and common association with poultry. Efforts were made to determine whether or not sparrows may become infected by cohabitation with infected chickens or, when infected, may transmit ND to susceptible chickens.

The literature shows that sparrows are susceptible to ND and the subject has been recently reviewed elsewhere.[2]

MATERIALS

Sparrows were collected on the premises of the Department of Veterinary Science of Purdue University, West Lafayette, Ind. Mature sparrows were defined as having mature plumage patterns and beaks which were completely cornified. Immature sparrows were of somewhat smaller size, with plumage patterns indicative of immaturity, and had incompletely cornified beaks.

Chickens were hatched from eggs from a White Leghorn flock and were raised in isolation that precluded the occurrence of recognizable disease.

The GB strain* of Newcastle disease virus (NDV) was used. The virus was passed in eggs two or three times prior to use.

Fertile eggs were obtained from the White Leghorn flock previously mentioned and were incubated at 37 C. from ten to twelve days prior to use. These embryonating eggs are referred to simply as "eggs" in this paper.

Chicken red blood cells in 1 per cent concentration in phosphate-buffered (pH 7.0) sodium chloride solution (0.85 %) were used in hemagglutination (HA) and hemagglutination-inhibition (HI) tests.

Antiserum for NDV for use in HI tests was obtained from chickens in response to the GB strain.

Aërosol exposure chambers were made of ¼-in. wire cloth cages of 0.44-cubic-foot volume, covered with heavy paper, and fitted with a truncated cone of the paper for the admission of the aërosol. The Devilbiss nebulizer No. 40** was used to nebulize the egg fluids containing the virus. This instrument is capable of producing particles which are nearly all below 5 μ in radius.[1]

The enclosure used in transmission studies to simulate natural conditions was constructed as previously described.[2]

METHODS

Aërosol exposure of the sparrows was accomplished in the following manner:

1) The sparrows were placed in the exposure chamber.

2) The nebulizer was filled with undiluted egg fluids containing the virus.

3) One hundred compressions of the bulb (ten in each thirty seconds) were made.

4) A five-minute interval was allowed to elapse.

5) One hundred compressions of the bulb were made again.

6) The sparrows were left in the chamber for thirty minutes. The volume expressed from the nebulizer into the chamber was approximately 0.85 ml. in all cases.

Tests for infectivity-neutralization (IN) antibodies, and for NDV in sparrow materials, were accomplished as described previously.[3] Tests for NDV in chicken materials were made in a fashion similar to that employed in the case of sparrow materials.

Tests for HA and HI antibodies were conducted on a glass plate. The tests were run simultaneously. The HA test consisted of mixing 1 drop of the suspected egg fluid with 1 drop of the red-cell preparation. The HI test consisted of the mixture of 1 drop of the suspected egg fluids, 1 drop of antiserum, and 1 drop of cells; the cells were added after the first two materials had been mixed.

Cohabitation of Immature Sparrows with Chickens Exposed to GB Virus. — Immature sparrows were placed in cohabitation with chickens exposed to NDV under conditions simulating nature, in that the population-space ratio afforded the chickens was that commonly found to be utilized for farm flocks. Ten, 5-week-old chickens were exposed intramuscularly to 10^5 l.d.$_{50}$ of virus and 10 to 10 l.d.$_{50}$. Twenty noninoculated chickens were added to prolong the episode of disease. Twenty-three immature sparrows were placed in the ex-

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* Obtained from North Central repository for Newcastle disease viruses, Department of Veterinary Science, University of Wisconsin, Madison.

** The Devilbiss Company, Toledo, Ohio.

closure with the chickens. Feed and water were supplied from the outside and the chamber was entered only to remove dead or moribund sparrows or chickens. Brain, liver, lungs, and intestinal contents of sparrows found dead were tested for the presence of NDV in eggs. The brains of 3 chickens selected at random were tested for virus. Paired serum samples from sparrows surviving thirty-five days were tested for IN antibodies and serum from surviving chickens was tested for HI antibodies.

RESULTS

Sparrows and chickens died in the pattern shown in table 1. Thirteen sparrows died during the trial; 3 died at unknown times, were decomposed when found, and were not further included in the data. The other 10 were found dead on the eleventh (2 birds), fourteenth, fifteenth (2 birds), sixteenth, seventeenth, twenty-first, and twenty-sixth (2 birds) days of the experiment. Isolations of virus from the sparrows were made, as shown in table 2, from the brains and lungs of 1 found dead on the twenty-first day, and from the liver of 1 and lungs of both sparrows found dead on the twenty-sixth day. Five paired samples of serum from the 10 sparrows remaining on the thirty-fifth day were found to contain demonstrable quantities of IN antibodies in 3 cases, ranging from 10 to 100 or more IN units, as shown in table 3. The morbidity among the chickens was 100 per cent and the mortality at thirty-one days was 87 per cent (35 of 40). All of the inoculated chickens were dead by the fourth day and 13 of the contact-exposed chickens died between five and twelve days postexposure; 2 others were dead by the twenty-fourth day. The virus was isolated from the brains of 3 chickens selected at random. The 5 chickens that survived showed nervous symptoms and high response to tests for HI antibodies.

Cohabitation of Chickens with Sparrows Exposed to NDV by an Aërosol Method.—Under the simulated natural conditions described for the preceding experiment, 40, 5-week-old chickens were placed in the enclosure with 9 mature sparrows which had been exposed to an aërosol of egg fluids containing $10^{8.0}$ $l.d._{50}$ per 0.1 ml. of virus (embryo titration). Six sparrows were placed in the enclosure on the first day and 3 were introduced on the fifth day of the experiment. The sparrows were withheld from the experiment for four days postexposure in an effort to circumvent the effect of contamination of their exteriors occurring coincident with exposure to the virus. Just prior to the placement of the first 6 sparrows with the chickens, each was washed in saline which was tested for virus by chicken inoculation. Five of the experimental chickens were tested for HI antibodies just prior to the experiment. Eight chickens of the same source were retained in isolation to give evidence as to whether or not incubative stages of ND existed when the trial was initiated.

Other than to remove experimental subjects as shown later, the chamber was not entered during the trial; feed and water were admitted by mechanical arrangements.

RESULTS

The chickens cohabitating with the exposed sparrows were first noticed to show respiratory distress on the sixth day of the trial; nervous symptoms were later shown by some of the chickens. Three of the chickens died on each of the eighth and ninth days, 9 on the tenth day, 7 on the eleventh day, and 10 on the twelfth day. Tracheal washings from 3 chickens were shown to contain NDV by tests in eggs and by the demonstration of HA activity of the egg fluids and its abolition by known NDV antiserum. The other 11 chickens, showing respiratory or nervous symptoms, or both, were killed on the twelfth day of the experiment.

Tests for HI antibodies in the chickens, just prior to the trial, showed them to be without such antibodies. At the termination of the experiment, serum from the 8 chickens, retained in isolation to determine

TABLE 1—Mortality Pattern of Chickens Inoculated with GB Strain of Newcastle Disease Virus and of Chickens and Sparrows Cohabitating with Them

Birds	Number died/ number exposed	Days until death						
		1-4	5-8	9-12	13-16	17-20	21-24	25-28
Inoculated chickens	20/20	20	....	....	....	....	....	....
Noninoculated chickens	15/20	....	5	8	1	....	1	....
Sparrows	13/23*	....	....	3	4	1	1	2

* Three sparrows died at unknown times.

whether or not the chickens were in the incubative stages of ND when the experiment was started, were found to be free of HI antibodies.

TABLE 2—Time of Death and Results of Tests for Virus in Materials of Sparrows Exposed to Infected Chickens

Days until death of sparrows	Brain	Liver	Lungs	Intestinal contents
11, 11, 14, 15, 15, 16, 17	.....	.....	.....	.....
21	positive	.....	positive	.....
26	.....	.....	positive	.....
26	.....	positive	positive	.....

Of the 9 mature sparrows exposed to GB virus, 7 died during the experiment and 2 were killed. The results of efforts to isolate virus from various materials from the 9 sparrows are shown in table 4. Of the materials tested, virus was isolated from five brains, five livers, eight lungs, and five intestinal contents. All 7 of the sparrows that died showed hemorrhagic enteritis. Efforts were made to isolate virus from fecal samples collected from sparrows 377, 379, 399, and 6 on the twelfth, thirteenth, and fourteenth days of the trial. Difficulties previously reported by the authors [3] were encountered when a lethal factor present in the feces killed the majority of eggs within twenty-four hours. Virus was not demonstrated in the fluids of these eggs or the fluids of those apparently not influenced by the lethal factor, nor did inoculations of these fluids in eggs reveal any virus.

Two of the sparrows (377 and 6) that died showed nervous symptoms which were noticed approximately forty-eight hours prior to death. The symptoms exhibited in 1 were clonic spasms of the cervical muscles which occurred about once each second and were continuous but somewhat variable in intensity. The second bird showed clonic opisthotonus at approximately five-second intervals. When held, the sparrows trembled in a fashion similar to that sometimes noticed in chickens affected with encephalomyelitis. Both of these sparrows remained capable of flight for several hours after the appearance of nervous symptoms.

Case Report

An immature sparrow was caught on the premises of the Department of Veterinary Science at Purdue University. It seemed to be ill as evidenced by its limited ability for flight. Immediately after capture it was killed and extracts of its liver, lungs, and intestinal contents were tested in eggs for NDV by the allantoic route.

Six of the 9 eggs used to test for virus were found dead at forty-five to sixty-three hours postinoculation; the other 3 survived until discarded at ten days postinoculation. All embryos inoculated with liver, 1 of 3 inoculated with lung, and 2 of 3 inoculated with intestinal contents died. The fluids of each of the 6 embryos that died were without HA activity by the plate test. However, the fluids of 1 of the eggs inoculated with intestinal contents was available for subculture later in 3 eggs. These eggs were found dead from forty-eight to sixty hours later. Extraembryonic fluids of these eggs were shown to have HA activity which was abolished by GB antiserum. Thus it would appear that NDV was demonstrated in the intestinal contents of an English sparrow *(Passer domesticus)* immediately upon collection from nature.

Discussion

The data presented support a belief that English sparrows *(Passer domesticus)* may be infected carriers of NDV. Transmission of NDV from infected chickens to immature sparrows and from infected sparrows to chickens under simulated natural conditions was demonstrated.

Transmissibility of NDV from chickens to immature sparrows was evidenced by isolation of virus from various tissues and

TABLE 3—Infectivity-Neutralizing (IN) Antibodies in Sparrows Surviving (35 Days) Simulated Natural Exposure to Newcastle Disease in Chickens

Serum sample*	Approximate IN units
1	0
2	0
3	10
4	100
5	100 or more

* Each sample was a composite from 2 sparrows.

demonstration of IN antibodies in the serum. Thirteen of 23 immature sparrows cohabitating with 40 infected chickens died during a 35-day experiment. Virus

TABLE 4—Time of Death and Results of Tests for Virus in Materials of Sparrows Exposed to an Aërosol of the Virus and to the Sparrow-Induced Infection in Chickens

Bird No.	Days post-exposure until death	Brain	Liver	Lung	Intestinal contents
1	7	.....	positive	positive	positive
2	10	positive	positive	positive	.....
3	10	positive	.....	positive	.....
4	11	positive	.....	positive	.....
5	11	.....	positive	positive	.....
377	12**	positive	positive	positive	positive
6	19**	positive	positive	positive	positive
379	15*	.....	.....	.....	positive
399	15*	.....	.....	positive	positive

** Showed nervous symptoms; * destroyed.

was isolated from 3 of the 13 sparrows that died; from the brain of 1, liver of 1, and the lungs of the 3 that died at twenty-one and twenty-six days of the trial. It is considered that this represents transmission, for although the isolation of virus from the lungs may have been due to contamination from the environment, isolation of virus from brain and liver would appear to represent invasion of these tissues. Infectivity-neutralization antibodies were found in three of five paired serum samples from the 10 sparrows which survived thirty-five days of the trial.

Spread of ND from NDV-exposed sparrows to chickens was evidenced by isolation of virus from tracheal washings of several chickens chosen at random among the 87 per cent that died and by the appearance of a syndrome similar to that frequently seen in NDV-infected chickens. Thirty-two chickens of 40 cohabitating with 9 sparrows died between eight and twelve days of the experiment. The disease was first noticed on about the sixth day when gasping and audible breathing were present. The presence of ND in the sparrows was established by isolation of NDV from five brains, five livers, eight lungs, and five intestinal contents of the 9 sparrows. Seven of the sparrows died during the trial and 2 were destroyed. All of the former showed hemorrhagic enteritis and the latter did not; however, virus was recovered from intestinal contents of the latter and from 3 of the former. Therefore, on the basis of virus isolations from intestinal contents no correlation was suggested between the presence of virus and hemorrhagic enteritis. Two of the 7 sparrows that died showed nervous symptoms which were similar to those noticed in ND in other species.

It has been previously reported that mature sparrows are susceptible to mild as well as virulent strains of NDV following intracranial or aërosol exposures [2,3] but that mature sparrows were refractory to infection by NDV when placed in close association with chickens undergoing a severe episode of the disease.[2] Evidence presented in this paper shows the susceptibility of immature sparrows under the latter similar conditions, thus giving evidence of greater susceptibility coincident with immaturity.

The fact that virus was recovered from sparrows as late as fifteen and nineteen days following aërosol exposure to NDV emphasizes the potential hazard that sparrows may represent as infected carriers of this disease. While it might be considered that the episode of disease in chickens presented a considerable continuing exposure of the sparrows, it is pointed out, as above, that mature sparrows were refractory under similar conditions to an episode of ND in chickens due to the same strain of virus. This suggests that the infection of the sparrows was due to the initial or aërosol exposure.

The results of these studies give increased significance to the suggestion that poultrymen take measures to exclude sparrows from their premises. Exclusion of these birds from the buildings in which poultry is continuously housed is a practicable procedure. Prevention of their nesting in farm buildings tends to minimize their population on the premises.

SUMMARY

Studies were made to determine whether or not Newcastle disease (ND) is transmissible from infected chickens to immature English sparrows and from infected sparrows to chickens. The following re-

sults are interpreted to signify that under simulated natural conditions such transmission was possible with the GB strain of Newcastle disease virus (NDV) and that natural occurrence of NDV in a sparrow has been observed.

1) Virus was isolated from lungs, liver, or brain of 3 of 23 immature sparrows that died at twenty-one and twenty-six days of cohabitation with infected chickens, and infectivity-neutralizing antibodies were demonstrated in three of five serum samples of sparrows that survived thirty-five days.

2) Chickens cohabitating with aërosol-infected sparrows under simulated natural conditions became infected with NDV as evidenced by the development of an ND-like syndrome and isolation of virus from tracheal washings. Virus was isolated from 7 of 9 sparrows that died seven to nineteen days following the initial exposure and from the other 2 which were killed at fifteen days.

3) Isolation of NDV from the intestinal contents of a sparrow which had acquired the virus in nature is reported for what is believed to be the first time.

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Immunization of Chickens Against Newcastle Disease by Formalin-Inactivated Vaccine

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PRELIMINARY experiments[1] have shown that a single injection of formalin-inactivated Newcastle disease vaccine results in a partial immunity. However, some data indicated that a greater immunity to challenge inoculation followed 2 doses of vaccine given three months apart. It was the purpose of this investigation to determine the optimal interval between 2 doses to obtain the greatest degree of immunity.

PROCEDURE

Six groups of 60 White Leghorn chicks of the same age, and raised in one house under isolation, were used in this experiment. All chicks to be vaccinated were given the first dose of 0.5 ml. of vaccine at 3 weeks of age. Thereafter, the groups were revaccinated with 1.0 ml. at different intervals from two to seventeen weeks after the initial dose (table 1). An alumina-gel, formalin-inactivated, commercial vaccine was used. Each group was challenged with virulent virus four months after the last dose of vaccine.

Two methods of challenge were used, namely, intramuscular and intranasal. Following intramuscular challenge with 0.1 ml. of 1:100 dilution of infected allantoic fluid, the criteria of infection after challenge were paralysis or death. The criterion of infection following intranasal inoculation of 0.05 ml. of a 1:100 dilution of infected allantoic fluid in each nostril was isolation of virus from the blood, spleen, or lung on the fourth day after challenge.

Prior to challenge a number of chickens were bled to obtain serums for serum antibody testing. The hemagglutination-inhibition (HI) test was done as previously described.[1] The virus infectivity-neutralization test in embryonating eggs was done according to standard procedure.[2]

The challenge strain of Newcastle disease virus was GB-Texas-1948. The inoculum consisted of pooled allantoic fluid from embryos previously inoculated with lung suspension from an infected bird. The pool was frozen at —30 C. until needed. A second pool was prepared similarly and was used for the last two challenge groups. During the course of the challenge experiments, the challenge virus was titrated in 7-week-old chicks three time to determine the chicken-infective dose. This was done by inoculating 6 to 8 chicks with one of four tenfold dilutions of virus intranasally and determining infection by virus isolation from the lung and spleen at ninety-six hours after inoculation. The first titration, done prior to challenge of the first group, revealed that 0.1 ml. contained $10^{5.9}$ chicken-infective doses. The second titration, prior to the third challenge, revealed $10^{6.4}$ chicken-infective doses per 0.1 ml. The third titration, after the final challenge with the second pool, revealed $10^{6.9}$ chicken-infective doses per 0.1 ml. of inoculum. On this basis the challenge dose inoculated was approximately 10,000 chicken-infective doses.

TABLE 1—Plan of Experiment

Group	Number of chickens: controls	Number of chickens: vaccinates	Age in weeks at time of 1st vaccination	Age in weeks at time of 2nd vaccination	Age in weeks at challenge
1	20	43	3	Not done	19
2	21	40	3	5	21
3	21	40	3	8	24
4	21	42	3	12	28
5	21	41	3	16	32
6	21	45	3	20	36

The results of challenge in each of the six groups are presented in table 2. Following a single dose of 0.5 ml. of vaccine at 3 weeks of age in group 1, the challenge results indicate that 25 per cent of 20 chickens were immune four months after vaccination when challenged by the intranasal route and infection determined by virus isolation. On the other hand, 82.6 per cent of 23 chickens were found immune when challenged intramuscularly. Paralysis, or death, were used as criteria of infection following intramuscular challenge. Approximately the same results were obtained in group 2 where vaccination was repeated two weeks after the initial dose.

In group 3, where the second dose of vaccine was given five weeks after the initial dose, the results of the intranasal challenge indicate 47.4 per cent of 19 chickens resisted infection, while the results of the intramuscular challenge indicate 100 per cent of 18 chickens survived.

Chickens in group 4 were given the second inoculation nine weeks after the initial

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dose and, as in the other groups, challenged four months after revaccination. Of 18 challenged intranasally, 16 (83.4 %) resisted the challenge on the basis of virus isolation from the tissues. Of 14 challenged intramuscularly, all survived and had no signs of Newcastle infection.

In group 5, where the second dose of vaccine was given thirteen weeks after the initial dose, 80 per cent of 20 chickens resisted the intranasal challenge. Following the intramuscular challenge, 93.7 per cent of 16 chickens were immune.

In group 6, those vaccinated resisted the challenge completely and the results of the serum testing revealed higher antibody values than were obtained from serums of group 5.

The results reveal a greater resistance in group-4 chickens than in group 5, despite the higher antibody level in serums from group-5 chickens. This difference in challenge results is possibly the result of the change in challenge pools of virus. Group 4 was challenged with a pool prepared at the beginning of the challenge work. This pool also thawed in the freezer, due to mechanical failure, a week prior to challenging group 4. The chickens in groups 5 and 6 were challenged with a second pool prepared prior to challenge of group 5 chickens.

With the exception of 1 control chicken in group 4, all other controls challenged intramuscularly had signs of paralysis, and usually clonic spasms, followed by death. This confirms the pathogenic nature of the GB strain of Newcastle disease virus. Of the 60 control chickens challenged intramuscularly, 42 had paralytic symptoms or were dead on the fourth day, 11 were paralyzed on the fifth day, 6 had paralytic symptoms on the sixth day and, as mentioned above, 1 remained normal. Of the 60 controls inoculated intranasally, none had signs of Newcastle disease on the fourth day when killed, but all were infected as indicated by positive virus isolation from the heparinized heart blood of each bird.

While the results of the virus infectivity-neutralization tests are somewhat irregular (table 3), there is a definite trend of increasing antibody titer to parallel the increasing resistance to challenge in groups 3 to 6. The logarithm of the geometric mean of the serum-neutralization results in groups 1 through 6 were 1.3, 1.6, 2.4, 3.0, 3.9, and 4.4, respectively. The HI results were characteristically low with a high of 400 HI units. There was no correlation between HI results and the infectivity-neutralization results or resistance to challenge.

Discussion

The results indicate that a partial immunity was established following a single 0.5-ml. dose of inactivated vaccine and that the degree of immunity obtained following two injections of vaccine depends upon the interval between doses. It appears that the longer the interval, up to seventeen weeks, the better the immunity obtained. The data indicate that an interval of nine weeks should be allowed between doses of the vaccine to obtain a substantial degree of immunity.

TABLE 2—Results of Challenging Vaccinated and Control Chickens

Group	Vaccination interval (wk.)	Chickens Infected after Challenge/Number Challenged				Vaccinates immune (%)	
		Intranasal		Intramuscular			
		control	vaccinate	control	vaccinate	Intranasal	Intramuscular
1	none	10/10	15/20	10/10	4/23	25.0	82.6
2	2	10/10	13/17	10/10	3/19	23.5	84.2
3	5	10/10	10/19	10/10	0/18	47.4	100.0
4	9	10/10	3/18	9/10	0/14	83.4	100.0
5	13	10/10	4/20	10/10	1/16	80.0	93.7
6	17	10/10	0/18	10/10	0/18	100.0	100.0

The results corroborate the observations by others regarding the basic principle that an animal gives a more rapid and greater response to an antigen if the animal has had previous experience with that antigen, and if a sufficient time interval is allowed between the initial and subsequent dose. Barr and Glenny,[3] confirming previous work, found that tetanus-antitoxin values could be greatly increased if, after the preliminary injections, a rest period of

three to six months or more was given before hyperimmunization.

Murray *et al.*,[4] in immunizing against epidemic typhus, obtained results which

TABLE 3—Results of Testing Serums from Vaccinated Chickens

Group and vaccination interval (wk.)		Samples of serums	Virus Neutralization: Neutralizing doses	Virus Neutralization: Logarithm of geometric mean	Hemagglutination inhibition**
1	none	6	2-100*	1.3	neg.
			4-10		
2	2	9	1-1000	1.6	neg.
			2-100		
			3-10		
			3-<10		
3	5	9	1-10,000	2.4	not done
			4-1000		
			2-100		
			2-10		
4	9	12	1-100,000	3.0	2-200
			3-10,000		4-100
			3-1000		6-neg.
			5-100		
5	13	15	3-100,000	3.9	1-400
			8-10,000		1-200
			4-1000		3-100
					10-neg.
6	17	14	7-100,000	4.4	2-400
			5-10,000		7-200
			2-1000		4-100
					1-neg.

* Two serums positive for 100 neutralizing doses.
** Expressed as HI units (one HI unit is amount that will inhibit one hemagglutinating unit).

suggested that the longer the interval between primary course injections the higher the complement-fixing antibody values. He also observed that the primary stimulus lasted up to five or six years and enabled a single booster inoculation to stimulate a maximum response.

Barr and Jones[5] in using diphtheria toxoid in guinea pigs found that, by using a two-month instead of one-month interval between doses, the response to the second injection could be increased. Subsequently, the response decreased more slowly as the interval between injections was increased. In another experiment using a smaller dose, an interval of three months appeared to be optimal. These authors found that a small dose of adsorbed diphtheria toxoid may confer excellent potential immunity in guinea pigs which at no time had detectable circulating antitoxin after the injection.

Hanson *et al.*[6] found that 76 to 96 per cent of chickens which were vaccinated once with inactivated Newcastle disease vaccine survived an intramuscular challenge. In group 1, which was also vaccinated once, 83 per cent of the birds survived the intramuscular challenge. This is in contrast to a figure of 25 per cent protection against infection when chickens from the same group were challenged intranasally and the interpretation based on virus isolation. The infectivity-neutralization test found between ten and 100 neutralizing doses in group-1 serums. It would appear that the neutralizing capacity of the serums could be more closely correlated with a 25 per cent rather than the 83 per cent immunity.

These figures, of course, represent two different factors and perhaps should not be compared. One represents protection against detectable virus infection and the other survival following challenge. A partial immunity may, and frequently does, protect a bird against the lethal effects of the virus. In recent unreported experiments, 45, 4-week-old chickens, which were vaccinated with inactivated Newcastle disease vaccine at 2 days of age, survived an aërosol exposure of Newcastle disease virus which was lethal to each of 17 controls. However, all those vaccinated had definite tracheal râles indicating infection. This is an instance where a partial immunity permitted 100 per cent survival of the vaccinated chicks, although all of them had symptoms of Newcastle disease following challenge.

SUMMARY

Six groups of 60 chickens each were used to determine the immunizing effect of two doses of inactivated Newcastle disease vaccine given at different intervals. The initial dose of vaccine was given at 3 weeks of age and the second dose was given at intervals of 2, 5, 9, 13, and 17 weeks. One group served as once-vaccinated controls. Each group, along with unvaccinated controls, was challenged with virulent virus four months after the second dose of vaccine. An increased resistance to challenge was demonstrated with the increasing intervals between doses of vaccine. The data indicate that an interval of at least nine weeks, and preferably longer, should be allowed between doses of alumina-gel, formalin-inactivated Newcastle disease vaccine, to obtain a substantial degree of immunity.

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A Method of Evaluating Immunity Following Vaccination of Chickens with Inactivated Newcastle Disease Vaccine

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INCONCLUSIVE results on the establishment of immunity to Newcastle disease by inactivated vaccines are found in the extensive literature reviews by Beaudette,[1-5] Brandly,[6] and Brandly *et al.*[7] Some of these variations arise from the use of different virus strains, inactivating agents, and adjuvants in preparing the vaccine. Interpretation of immunity has been further complicated by the use of different routes of inoculation with various strains and doses of challenge virus. Better methods are needed to measure the immunity produced by Newcastle disease vaccines.

The method commonly used in challenging birds which have been vaccinated is to administer the challenge virus intramuscularly and to use survival versus death as the criterion of immunity. This method of measuring immunity does not appear to be adequate to accurately evaluate the immunity in chickens. In order to obtain more knowledge on the status of the immunity in chickens vaccinated with killed Newcastle disease vaccine, interpretation of challenge results in this investigation was based upon virus isolation from the blood, spleen, and lung at seventy-two or ninety-six hours following challenge inoculation. The basic objective was to determine the ability of the challenge virus to multiply in the tissues of the vaccinated chickens. If the virus was unable to propagate in the tissues following adequate intranasal challenge, the assumption was that the chicken was immune. Determinations of the concentration of virus in the tissues of vaccinated chickens compared to the concentration of virus in unvaccinated chickens following challenge might give information as to the degree of immunity.

METHODS AND MATERIALS

The chickens used were from the flock kept at the Veterinary Research Institute. Each vaccinated group, along with unvaccinated controls, was kept isolated from the rest of the flock and cared for by one caretaker who tended no other chickens.

The vaccine used was a commercial, aluminagel, formalin-inactivated vaccine purchased through regular retail outlets. The vaccine was administered according to recommendations by the vaccine producer. With the exception of the vaccine selected in the initial dose in trial 4, all the vaccine used was from a single producer.

Two challenge strains of Newcastle disease virus (NDV) were used. Iowa-228-1947 was a field strain isolated in Iowa. This strain was not titrated in chickens. GB-Texas 1948, obtained from Dr. E. R. Doll, was used in some groups as the challenge virus. Chorioallantoic fluid from eggs infected with this strain usually contained 10^8 chicken-infective doses based on titration in 7-week-old chickens when 0.1 ml. was given intranasally and the end point based on virus isolation. The birds were challenged by inoculation of 0.05 ml. of various dilutions of virus-infected allantoic fluid into each nostril of the birds. In most trials, the birds were bled and killed at ninety-six hours following challenge. The spleen and lungs were collected for virus isolation or virus titration. Blood samples were titrated in embryos on the day of collection, or the following day, using fivefold serial dilutions. This procedure was used for blood since previous experience had found only a relatively small amount of virus in the blood. Since a dose of 0.2 ml. was used for egg inoculation, the quantity of virus per milliliter listed in the tables is obtained by multiplying 5 times the end point dilution. The tissues were held in a frozen state until they could be titrated. Prior to virus titration the tissues were thawed, weighed, and tenfold dilutions made for embryo inoculation. Hemagglutination inhibition (HI) tests were done by the *beta* procedure. Ten hemagglutinating units of formalin-killed virus were used in each of a series of twofold dilutions of serum. The serum dilutions were prepared directly in the 0.5-ml. virus suspensions. Chicken red blood cells were used at a concentration of 0.25 per cent in 0.5-ml. units to detect specific inhibition. Strain 174-Iowa-1947 was used as antigen in the HI test.

PROCEDURE AND RESULTS

Trial 1. — Three-month-old pullets were vaccinated with 1 ml. of vaccine, intramuscularly. Unvaccinated controls were raised with the vaccinated birds. Some in the group were removed at intervals of two months and challenged with a 1 : 10 dilution of infected allantoic fluid contain-

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ing approximately 10^8 embryo lethal doses (e.l.d.) of strain 228 of Newcastle disease virus (NDV).

In table 1, the results of challenge are presented. Many of the vaccinated birds appeared to have some degree of protection. Although virus could be frequently isolated, it is evident that the tissues did not have as great a concentration of virus as the tissues from control chickens. In some birds, virus was isolated from the blood when virus could not be detected in the lung and spleen. Serums from all vaccinated birds and controls were negative for HI antibodies at the time of challenge.

TABLE 1—Titer of Newcastle Disease Virus (NDV) in Tissues of Chickens Seventy-Two Hours After Intranasal Challenge with 10^7 Embryo Lethal Doses (e.l.d.) of Strain 228

			e.l.d. of NDV per ml. tissue suspension		
Challenge	Bird No.		blood	lung	spleen
Two months after vaccination	Control	1	625	$10^{4.0}$	$10^{3.5}$
		2	3125	$10^{4.0}$	$10^{3.5}$
	Vaccinate	3	125	$10^{2.5}$	$10^{4.5}$
		4	0	0	0
		5	0	0	0
Four months after vaccination	Control	6	625	$10^{3.0}$	$10^{3.0}$
		7	625	$10^{3.0}$	$10^{3.0}$
	Vaccinate	8	125	0	0
		9	25	0	0
		10	25	0	10
		11	125	0	0
		12	0	0	0
		13	0	0	0
		14	0	0	0
		15	0	0	0
Six months after vaccination	Control	16	625	$10^{3.0}$	$10^{4.0}$
		17	625	$10^{3.0}$	$10^{3.5}$
		18	125	$10^{4.5}$	$10^{4.5}$
		19	125	$10^{4.5}$	$10^{4.5}$
		20	125	$10^{3.0}$	$10^{3.0}$
	Vaccinate	21	0	10	10
		22	0	50	10
		23	125	$10^{2.5}$	$10^{2.0}$
		24	5	0	0
		25	0	$10^{1.5}$	0
		26	5	0	0
		27	0	0	0
		28	0	0	0
Eight months after vaccination	Control	29	5	$10^{5.0}$	$10^{5.0}$
		30	125	$10^{3.5}$	$10^{5.5}$
		31	25	$10^{3.0}$	$10^{3.0}$
		32	25	$10^{4.0}$	$10^{2.0}$
		33	625	$10^{4.0}$	$10^{4.0}$
		34	25	$10^{3.0}$	$10^{2.0}$
	Vaccinate	35	5	$10^{5.0}$	50
		36	125	$10^{3.5}$	$10^{4.5}$
		37	5	0	0
		38	0	10	0
		39	0	0	0
		40	0	0	0

Trial 2.—The procedure in trial 1 was varied to study the effect of a smaller challenge dose. The challenge dose was a 10^{-5} dilution of allantoic fluid of strain 228 NDV.

The results of challenge are presented in table 2. The vaccinated birds withstood the smaller challenge dose for eight months at which time 3 out of 7 birds vaccinated failed to resist the challenge. One unvaccinated control, challenged at six months, failed to become infected, although all the controls in the experiment were negative for HI antibodies against NDV, as were those vaccinated.

Trial 3.—This trial was made to determine the efficacy of 2 doses of vaccine given two weeks apart. The chickens were 6 weeks old when the first injection was given. The challenge strain of NDV in this trial was GB-Texas-1948 and a 10^{-6} dilution of infected allantoic fluid was used, except that a 10^{-5} dilution was used for the birds challenged at nine months after vaccination.

The results of challenge are presented in table 3. At two months the vaccinated chickens resisted the challenge although at four months after vaccination virus was isolated from 2 of 8 vaccinated birds. Six months after vaccination, virus was isolated from half those vaccinated and at nine months none of the birds resisted the increased challenge dose. Serums from all

TABLE 2—Titer of Newcastle Disease Virus (NDV) in Tissues of Chickens Ninety-Six Hours After Intranasal Challenge with 10^5 Embryo Lethal Doses (e.l.d.) of Strain 228

			e.l.d. of NDV per ml. tissue suspension		
Challenge	Bird No.		blood	lung	spleen
Two months after vaccination	Control	1	125	$10^{3.5}$	$10^{4.5}$
		2	25	$10^{2.5}$	$10^{3.5}$
	Each vaccinate	3-8	0	0	0
Four months after vaccination	Control	9	125	$10^{3.7}$	$10^{2.0}$
		10	25	$10^{2.5}$	$10^{2.7}$
	Each vaccinate	11-16	0	0	0
Six months after vaccination	Control	17	125	$10^{4.7}$	$10^{3.7}$
		18	0	0	0
	Each vaccinate	19-24	0	0	0
Eight months after vaccination	Control	25	25	0	$10^{3.0}$
		26	25	$10^{3.5}$	$10^{3.5}$
		27	125	$10^{3.5}$	$10^{3.5}$
		28	25	$10^{4.7}$	$10^{3.7}$
		29	125	$10^{4.0}$	$10^{3.0}$
	Vaccinate	30	25	$10^{2.5}$	$10^{3.0}$
		31	5	$10^{3.0}$	0
		32	5	0	0
		33	0	0	0
		34	0	0	0
		35	0	0	0
		36	0	0	0

those vaccinated and from the controls were negative for HI antibodies at the time of challenge.

Trial 4.—In this group, two doses of vaccine were given three months apart. The first dose was injected at 3 months of age and the vaccine used was the same type but from a different commercial company. The second dose of vaccine was given when the chickens were 6 months of age. The challenge virus was a 10^{-3} dilution of infected allantoic fluid of GB-Texas-1948.

TABLE 3—Titer of Newcastle Disease Virus (NDV) in Tissues of Chickens Ninety-Six Hours After Challenge with 10^5 Embryo Lethal Doses (e.l.d.) of Strain GB

			e.l.d. of NDV per ml. tissue suspension		
Challenge	Bird No.		blood	lung	spleen
Two months after 2nd vaccination	Control	1	125	$10^{6.5}$	$10^{6.3}$
		2	625	$10^{5.5}$	$10^{5.9}$
	Each vaccinate	7-9	0	0	0
Four months after 2nd vaccination	Control	10	125	$10^{6.5}$	$10^{5.5}$
		11	625	$10^{6.3}$	$10^{4.9}$
	Vaccinate	12	25	0	0
		13	0	25	0
	Each vaccinate	14-19	0	0	0
Six months after 2nd vaccination	Control	20	3125	$10^{5.5}$	$10^{5.9}$
		21	25	$10^{3.5}$	$10^{2.5}$
	Vaccinate	22	0	$10^{2.9}$	0
		23	5	0	0
		24	25	0	0
		25	5	0	0
	Each vaccinate	26-29	0	0	0
Nine months after 2nd vaccination Challenge of 10^7 e.l.d.	Control	30	625	$10^{6.5}$	$10^{4.5}$
		31	625	$10^{7.3}$	$10^{6.9}$
		32	625	$10^{7.3}$	$10^{6.5}$
	Vaccinate	33	25	$10^{2.9}$	$10^{2.6}$
		34	5	0	0
		35	625	$10^{5.5}$	0
		36	625	$10^{4.5}$	0
		37	625	$10^{4.5}$	$10^{3.5}$
		38	125	$10^{3.5}$	0
		39	625	$10^{4.5}$	$10^{3.5}$
		40	625	$10^{5.5}$	10

Results are presented in table 4. Two months after the first dose of vaccine, only 2 of 7 vaccinated birds resisted the challenge. One month after revaccination, 1 of 7 failed to resist challenge. At three months after the revaccination, all of the 8 vaccinated birds resisted the challenge. At eight months, 1 of 10 failed to completely resist infection. The HI results of the controls were negative, while serums from some of the twice-vaccinated chickens gave inhibition in the 1:20 and 1:40 dilution of serum. None of the serums tested greater than 400 HI units (40 x 10 hemagglutinating units).

Trial 5.—In this group, the first dose was given at 3 months of age and revaccination done three months later. The challenge strain was the GB-Texas and a 10^{-2} dilution was used.

The results are presented in table 5. No titrations were done and the results are given as positive or negative virus isolations. Challenge at three months after the initial dose revealed that half of the birds were susceptible to challenge. One month after the revaccination, all of 6 vaccinated chickens resisted challenge. Eight months after revaccination the 14 vaccinated chickens resisted challenge. The HI results were similar to those obtained in trial 4. The virus-infectivity-neutralization tests with some serums from twice-vaccinated birds revealed at least 10,000 neutralizing doses.

DISCUSSION

In these experiments, results following challenge with NDV was based on isola-

TABLE 4—Titer of Newcastle Disease Virus (NDV) in Tissues of Chickens Ninety-Six Hours After Intranasal Challenge with 10^5 Embryo Lethal Doses (e.l.d.) of Strain GB

			e.l.d. of NDV per ml. tissue suspension		
Challenge	Bird No.		blood	lung	spleen
Two months after 1st vaccination	Control	1	125	$10^{4.7}$	$10^{3.7}$
		2	625	$10^{6.9}$	$10^{5.5}$
		3	625	$10^{5.0}$	$10^{5.0}$
	Vaccinate	4	0	$10^{4.7}$	$10^{4.5}$
		5	125	50	0
		6	0	$10^{2.0}$	50
		7	125	$10^{3.0}$	$10^{2.0}$
		8	125	50	$10^{2.0}$
	Each vaccinate	9-10	0	0	0
Revaccinated 3 months after first dose					
One month after 2nd vaccination	Control	11	25	$10^{3.7}$	$10^{4.7}$
		12	125	$10^{3.5}$	$10^{3.5}$
		13	5	$10^{3.7}$	$10^{3.9}$
	Vaccinate	14	5	0	0
	Each vaccinate	15-20	0	0	0
Three months after 2nd vaccination	Control	21	125	$10^{5.0}$	$10^{4.0}$
		22	125	$10^{6.9}$	$10^{4.5}$
		23	625	$10^{4.5}$	$10^{4.0}$
		24	125	$10^{5.0}$	$10^{5.0}$
	Each vaccinate	25-32	0	0	0
Eight months after 2nd vaccination	Control	33	3125	$10^{6.0}$	$10^{5.0}$
		34	625	$10^{5.0}$	$10^{4.0}$
		35	125	$10^{5.0}$	$10^{5.0}$
		36	625	$10^{4.0}$	$10^{4.0}$
	Vaccinate	37	25	0	0
	Each vaccinate	38-46	0	0	0

tion of virus from lung, spleen, and blood. The method was cumbersome at the beginning since virus titrations of the tissues were made. However, as carried out in trial 5, where only a positive or negative virus isolation was desired, the method was readily workable and was considered to be a reliable means of measuring the degree of immunity produced.

Methods other than virus isolation could possibly be used to detect infection following challenge in vaccinated chickens. A drop in egg production or feed consumption might be indicative of infection in laying birds. Respiratory symptoms following challenge by aërosol would indicate infection with the virus. These methods would be a more critical test of immunity than would survival following challenge.

It appears that a single dose of aluminagel, formalin-inactivated Newcastle disease vaccine, gives a partial immunity in chickens, but it does not give the necessary immunity to fully protect a flock. That a partial immunity is present for as long as eight months in some birds is evinced by the lower concentration of virus in the tissues following challenge as compared to the unvaccinated controls. This was obvious throughout the experiment. Even though the chickens had negligible serum antibody levels, they possessed a degree of immunity sufficient to keep the concentration of the virus to a low level in some of the birds. The data also indicate that the size of the challenge dose could influence the results of challenge experiments.

Chickens vaccinated twice within an interval of two weeks appear to have little, if any, advantage over birds vaccinated once. However, when chickens were given two doses of vaccine at an interval of three months between doses a substantial degree of immunity resulted which lasted at least eight months after the last dose of vaccine.

There was little correlation between HI results and challenge results. The HI antibody level is characteristically low in chickens which have been vaccinated with inactivated vaccines. Even in serums from chickens vaccinated with two doses given three months apart, a low HI antibody level is observed, although such serums may have a fairly high infectivity-neutralizing antibody level.

TABLE 5 — Newcastle Disease Virus (NDV) Isolation from Tissues of Chickens Ninety-Six Hours After Intranasal Challenge with 10^6 Embryo Lethal Doses (e.l.d.) of Strain GB

Challenge	Bird No.	NDV isolations		
		blood	lung	spleen
Three months after 1st vaccination	3 controls	+	+	+
	3 vaccinates	0	0	0
	3 vaccinates	+	+	+
Revaccinated 3 months after first dose				
One month after 2nd vaccination	4 controls	+	+	+
	6 vaccinates	0	0	0
Eight months after 2nd vaccination	9 controls	+	not done	not done
	14 vaccinates	0	0	0

SUMMARY

The method used to measure the immunity was to challenge chickens by intranasal inoculation and to determine the presence or absence of virus in the blood, spleen, or lung seventy-two or ninety-six hours later. Failure to isolate virus from the tissues was considered evidence of immunity. Preliminary experiments were carried out to evaluate the immunity in five groups of chickens following the use of formalin-inactivated Newcastle disease vaccine. The study revealed that a single dose of vaccine given to 3-month-old chickens induced a partial immunity. A second dose of vaccine given two weeks after the first injection did not definitely enhance the immunity. However, two doses given three months apart did result in a substantial degree of immunity which lasted at least eight months after the last dose of vaccine.

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The Behavior of a Transmissible Lymphoid Tumor in the Tissues of Chickens Inbred for Resistance and Susceptibility to Spontaneous Lymphomatosis

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SELECTIVE breeding for resistance to lymphomatosis in chickens has been shown to be effective in reducing the number of deaths from the disease. By raising only the progeny of birds over 500 days of age, Hutt and Cole [1] demonstrated that losses could be reduced from 35 per cent to 4 per cent, over a period of ten years' selection.

Heisdorf *et al.*[2] demonstrated that inbred resistance to lymphomatosis could be overcome by direct inoculation of the causative agent into susceptible and resistant lines of chickens.

The present study was designed to demonstrate the behavior of transplantable lymphoid tumor cells in the tissues of inbred resistant and susceptible chickens.

MATERIALS AND METHODS

The Origin of Strain RPL No. 16 Tumor.—The original tumor specimen was taken from the liver of a naturally occurring case of lymphomatosis in a White Leghorn hen at the Regional Poultry Research Laboratory, East Lansing, Michigan. A complete history of the tumor until it was received for the present study was reported by Burmester *et al.*[3]

Genetic Constitution of the Birds.—The U. S. Regional Poultry Research Laboratory at East Lansing maintains White Leghorn chickens for experimental studies on lymphomatosis. Breeding lines are selected toward both natural resistance and susceptibility to the disease. Line 15 susceptible and line 6 resistant were made available for this study. In 1947, line 15 showed a mortality rate of 29 per cent and line 6 a mortality rate of 8 per cent from the naturally occurring disease to the age of 400 days.[4]

Preparation of the Inoculum.—The tumor tissue used had been preserved at the Regional Poultry Laboratory according to a method described by Burmester *et al.*[5] The tumor sample received for this study had been stored for sixteen months.

The specimen was removed from a block of dry ice and thawed in the ampule under running tap water. A piece of thawed tumor tissue weighing approximately 5 Gm. was pressed through a tumor mincer and the resultant mash suspended in 100 cc. of normal saline solution. Cell counts of this suspension were obtained by dilution in a white blood cell pipette and computation on the Neubauer scale. The suspension was then diluted to yield approximately 10,000 cells in 0.5 cc.

Dose.—Approximately 10,000 cells per dose were used.* It was conceivable that the natural resistance of the birds might have a more favorable chance to exert its influence when a smaller number of cells was used.

Route of Injection.—The intramuscular route was chosen for the site of inoculation since it could be easily defined and since 100 per cent of takes had been obtained on previous trials. Inoculations were made into the left breast muscle near the wing joint.

Procedure.—A total of 107 line 6 (resistant) and 91 line 15 (susceptible) 11-day-old chicks were used in the experiment. Sixty-five chicks from each group were inoculated with tumor cells. Following inoculation, birds from each line were killed in groups of 5 at twelve hours, twenty-four hours, and daily thereafter for ten days. Ten birds from each line were not killed but allowed to terminate in death from the disease, to serve as inoculation controls. Uninoculated controls were killed in groups of 5 at twelve hours, three days, five days, and seven days. Twenty-two line 6 and 6 line 15 birds were set aside to observe for naturally occurring tumor development during the experimental period.

RESULTS

One of the resistant and 2 of the susceptible birds in the inoculation control group survived the transplant. A 2- to 3-cm. tumor arose at the site of inoculation in all 3 of these birds, reaching the peak of growth in three weeks. These tumors

This study was completed in partial fulfillment of the requirements for the degree of doctor of philosophy from the University of Wisconsin, 1950, and was made possible under a special cancer fellowship from the National Cancer Institute, Bethesda 14, Md.

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* Previous trials using nearly 1,000,000 cells had resulted in rapid necrosis at the inoculation site.

appeared circumscribed in contrast to the others in which growth was more diffuse throughout the muscle. Within two months these tumors had completely regressed. The

Fig. 1—RPL No. 16 tumor ten days following inoculation of cells into the pectoral muscles.

remaining 7 birds of the inoculated control group were dead by the eleventh day. There were no deaths from lymphomatosis among the birds set aside for observation of spontaneous tumor development.

Gross Observation

Site of Inoculation.—Gross swelling of the breast muscles at the site of inoculation was seen on the fourth day. Growth was diffuse and the tumor was not sharply demarcated from the muscle tissue. Swelling had increased considerably by the fifth day and a gelatinous material was seen in the subcutaneous tissues. The swelling and edema progressively increased until death of the birds. At the time of death, the tumor had caused an increase in the size of the muscle to at least four times its original dimensions, and a rounded mass, approximately 2 cm. in diameter, had developed at the injection site (fig. 1 and 2). There was no ulceration. On sectioning, the tumor appeared homogeneous and pearly grey with necrotic areas near the center. No essential difference was seen in the appearance of the tumor in either susceptible or resistant birds.

Liver.—The first change was observed on the sixth day when there was slight enlargement and congestion. Congestion was more severe on the seventh day and by the eighth day small hemorrhages were seen on the surface. Cut edges bulged and the hemorrhagic areas extended deeply into the parenchyma. No solid tumor masses were observed. Livers of birds dying subsequently showed essentially the same gross characteristics.

Proventriculus.—There was slight enlargement on the sixth day and at the time of death the organ was about one-third larger than normal. The swelling was confined to the mucosal lining and extended down to the muscular layer.

Gross abnormalities were not seen in the remainder of the organs.

Microscopic Observations

Site of Inoculation.—Tumor cells from the inoculum were observed lying between the muscle bundles in the tissues taken at twelve hours. At twenty-four hours, there was some evidence of proliferation, but the tumor cells were confined to the intermuscular septums. However, two sections showed beginning invasion between muscle fibers with the cells tending to aggregate

Fig. 2—Cross section of tumor in figure 1, showing central necrosis and hemorrhage.

about blood vessels. At forty-eight hours, invasion had progressed and small tumor foci appeared around the blood vessels. Mitoses of the tumor cells were numerous. On the fourth day, small areas of necrosis were seen in the larger tumor masses. By the sixth day, there was considerable necrosis of tumor tissue, particularly around the blood vessels. In one section, a blood vessel appeared to be invaded and the lumen occupied by tumor cells (fig. 3). This bird, one of the resistant group, also showed a few cells in the blood vessels of the kidney, which appeared identical with those of the original tumor. This was the only metastasis observed at six days either in the remainder of this group or in the susceptible group. Inflammatory reaction was not seen in the tissue surrounding the tumor up to this time. By the seventh and eighth day, the breast muscles were extensively invaded, necrotic foci were numerous, infiltrations of neutrophils appeared, and minimal fibroblastic activity was seen. Coincident with the changes observed locally, increasing numbers of tumor cells were found in the sinusoids of the liver and in the blood vessels of the kidneys.

The tumor cells appeared to vary in shape when compressed within blood sinusoids and between muscle fibers (fig. 4). In loose tissues or when seen free at the margins of the section, the cells appeared large and rounded.

No appreciable differences were detected in the appearance of the tumor at the site of inoculation in either resistant or susceptible birds.

Lungs.—Foci of lymphoid tissue were found beneath the mucosa of the bronchioles in both susceptible and resistant birds, including inoculated and control animals. The cells appeared to be mature, small lymphocytes, although an occasional section showed proliferating nodules within these lymphoid areas. The latter appeared more prominent at the junction with secondary bronchi. They were seen more frequently in the resistant birds. In the terminal stages of the disease, small groups of tumor

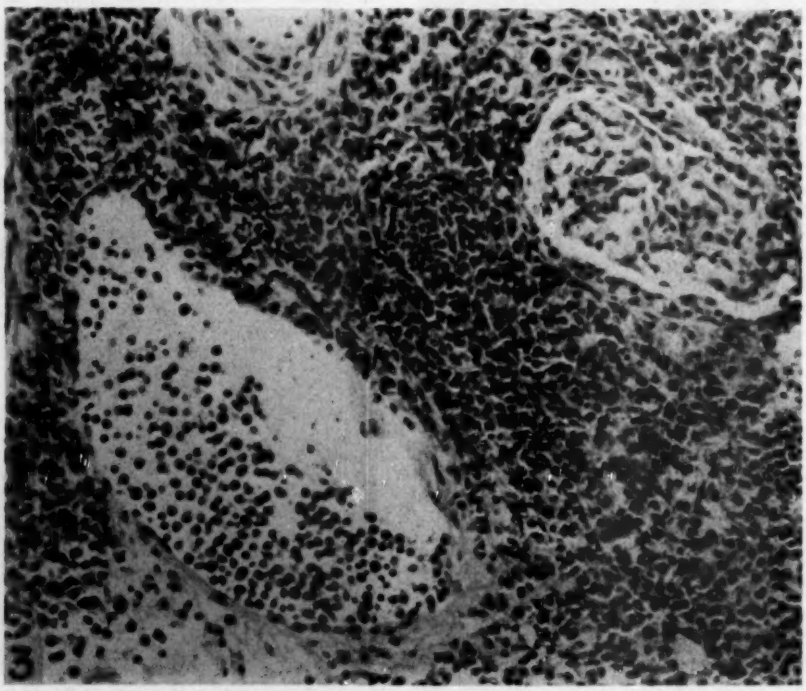

Fig. 3—Section of muscle showing tumor cells aggregated about blood vessels and nerve. Note break-through in wall of large vein and presence of tumor cells in the lumen; also invasion of nerve by tumor cells. Hematoxylin-eosin. x 320.

cells were observed in the septums of blood vessels of the lungs.

Liver.—Lymphoid accumulations, varying in size from a few scattered cells to large aggregations amounting to several hundred cells, were seen surrounding blood vessels (fig. 5). These occurred in both those inoculated and in the controls of the susceptible and resistant birds but were most numerous in the latter. As the tumor growth at the site of inoculation progressed, lymphoid tissue became more abundant around the portal vessels, particularly in the resistant birds. Periportal lymphoid accumulations in the control birds of both lines were essentially similar in birds killed at the beginning and at the end of the experiment. They were more abundant in the resistant than in the susceptible line. These observations suggested that the increased extent of this tissue in the tumor-bearing animals could be a systemic effect of the disease.

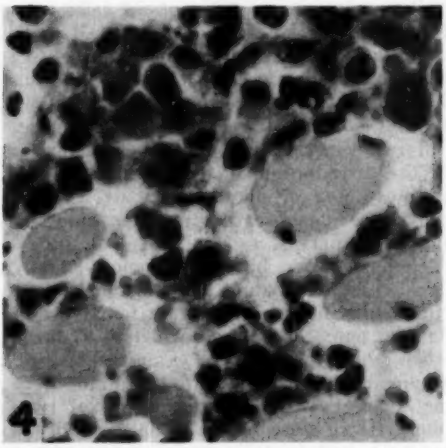

Fig. 4—High power view of tumor cells invading skeletal muscle. Hematoxylin-eosin. x 410.

On the eighth day following inoculation, cells identical with those of the inoculum were seen in the hepatic sinusoids. These cells became more numerous at later stages and in the terminal stages of the disease they were packed in the sinusoids (fig. 6). There were extensive hemorrhages and areas of necrosis in the liver parenchyma at the time of death (fig. 7).

Kidney.—Occasional lymphoid foci were seen surrounding blood vessels in the cortex of the kidney in both resistant and sus-

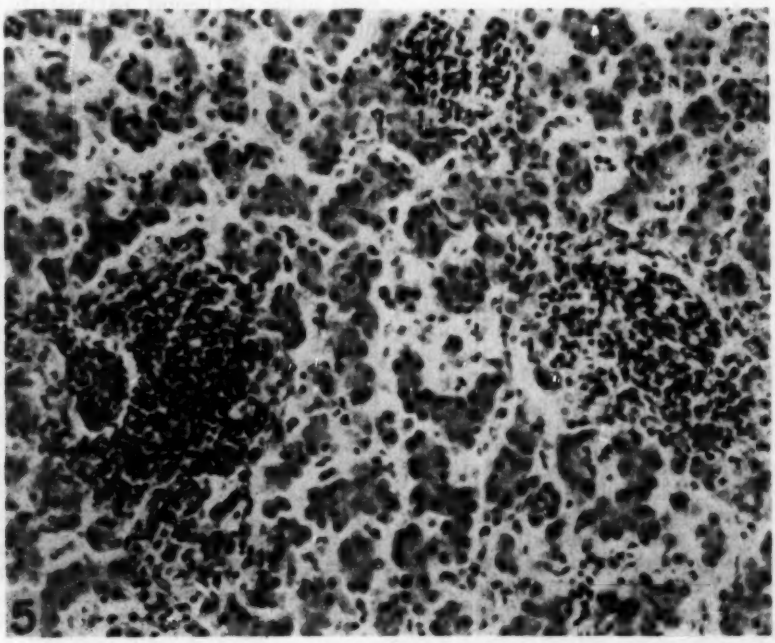

Fig. 5—Aggregates of reactive lymphocytes around portal vessels. Hematoxylin-eosin. x 315.

ceptible birds, in those inoculated and in the controls. There was no numerical increase or change in the character of these foci during progressive tumor development

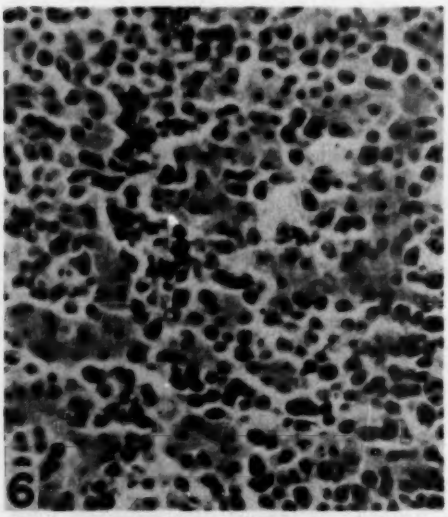

Fig. 6—The liver, showing accumulation of tumor cells in sinusoids during the terminal stages of the disease. Hematoxylin-eosin. x 380.

in either inoculated or control birds. The first metastatic focus of tumor cells appeared in a blood vessel of the kidney in a resistant bird on the sixth day following inoculation. On the seventh, eighth, and ninth days these foci were observed more frequently and terminally there was a diffuse infiltration of cells between the cortical tubules in both lines of inoculated birds. (fig. 8).

Proventriculus.—The uninoculated controls, in both resistant and susceptible birds, showed patches of mature lymphoid cells under the mucous membrane of the central canal and occasional small foci in the submucosal glandular crypts. There was no change in the appearance or quantity of these lymphoid accumulations in either control series. In the terminal stages of inoculated birds, large numbers of tumor cells appeared in the submucosal glandular areas. The cells appeared to be transported via the small vessels originating in the periphery between the glandular layer and the muscle coats. They were packed into these vessels in a linear fashion, and occasionally large masses of cells were seen near the tip of the villus (fig. 9). This infiltration occurred in essentially the same manner in both resistant and susceptible birds.

Pancreas.—Foci of lymphoid cells were found in the pancreas of nearly all birds examined, including controls. These foci varied from a few cells to extensive areas 15 to 20 μ in diameter; they were usually around blood vessels. There was no essential difference in the number, the character, or in the distribution of these lymphoid foci in inoculated or control animals. Tumor cells were not observed in any of the sections of pancreas.

Gonads.—The birds were not equally divided as to sex since it is extremely difficult to distinguish male from female at 11 days of age. Sex was recorded in 97 birds when the tissues were examined microscopically. Thirty-eight were found to be males and 59 were females. Foci of lymphoid tissue were found in one testicle (a control) and in two ovaries (one control and one inoculated). The cells in these accumulations were mature lymphocytes. The gonads were not involved in tumor metastases.

Intestine.—Large aggregations of lymphoid tissue were seen underlying the mucosal lining of the gut, particularly at the ileocecal junction. Sections were taken at

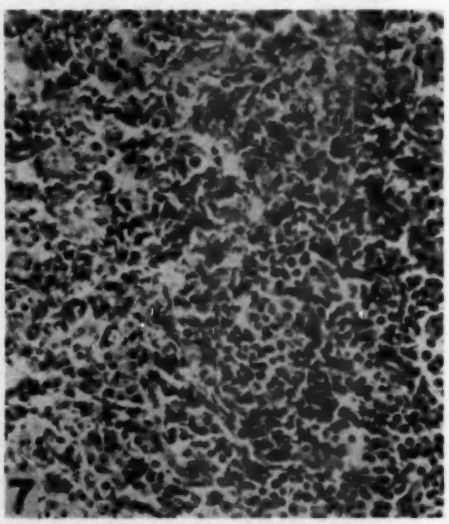

Fig. 7—Congestion and hemorrhage in the liver during the terminal stages of the disease. Hematoxylin-eosin. x 235.

this site in all birds. The appearance of this lymphoid tissue was uniform in both inoculated and control birds. The intestines were not involved in tumor metastases.

Spleen.—The reaction in the spleen was similar to that in the other lymphoid tissues of the body. Coincident with the growth of the tumor transplant, proliferating centers of lymphocytes became more numerous. Differences were not observed between resistant and susceptible birds. Occasionally, groups of cells resembling those of the tumor were seen in the sinusoids during the terminal stages of the disease.

Adrenal Gland.—Neither lymphoid foci nor tumor cells were demonstrated in either control or inoculated birds.

Bone Marrow.—There was a mild reactive response in the marrow of all inoculated birds. In the terminal stages of the disease in both resistant and susceptible birds, large cells resembling those of the tumor were seen in the sinusoids.

Thymus.—The thymus appeared normal in birds of all groups.

Heart.—On one occasion a small focus of lymphoid cells was found in the myocardium. Tumor cells were not seen in the heart tissues of any of the birds.

DISCUSSION

In the fowl, the lymphoid tissues are not organized into a system of nodes but rather are scattered throughout the body with extensive accumulations being found in the submucosa of the digestive tract, occasionally under the bronchial epithelium, skin, in the liver, pancreas, kidneys, gonads, and along nerves and blood vessels. The only truly organized lymphoid organs are the spleen, thymus, and bursa of Fabricus.[6]

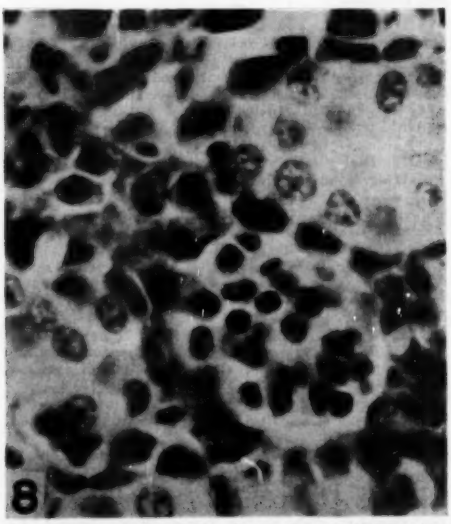

Fig. 8—Section of kidney showing infiltration of tumor cells between the cortical tubules. Hematoxylin-eosin. x 410.

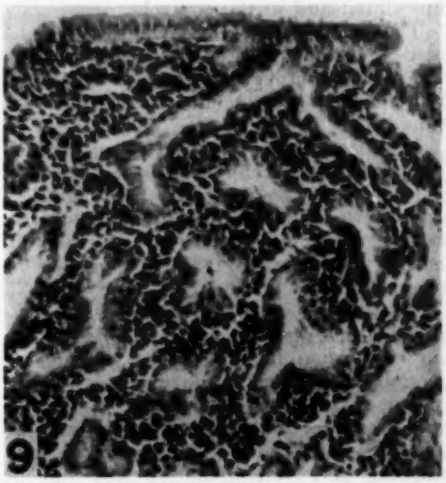

Fig. 9—Invasion of tumor into the proventriculus. In the terminal stages of the disease, the vessels of the tunica propria in the glandular mucosa were engorged with tumor cells. Hematoxylin-eosin. x 200.

The significance of lymphoid areas in the different organs of the body has been variously explained. Dantchakoff[7] regards them as being normal, while Lucas believes that, in other than the thymus, spleen, and bursa of Fabricus, they are abnormal in the sense that they are a response to external stimuli. In this study, the cellular elements of these accumulations had the appearance of normal circulating lymphocytes. Qualitatively, at least, no difference in these lymphoid areas was observed in control or inoculated birds in either susceptible or resistant lines.

There was no evidence that the normal lymphoid foci became neoplastic, although there was some variation in their distribution and extent in different individuals. More extensive areas were seen in the resistant birds than in the susceptible in those inoculated and in the controls. There was a greater increase in the size and num-

ber of these lymphoid areas in the livers of inoculated resistant birds as compared to controls in this line, in contrast to the susceptible group in which they were fairly constant in size and distribution in those inoculated and in the controls.

The distribution of lesions in spontaneous cases of lymphomatosis has indicated that the foci of lymphoid tissue in the body of the chicken were the sites of origin of the tumors.

It appears that with the lymphoid tumor, RPL No. 16, the donor cells were propagated in the tissues of the recipient host and grew until death resulted from metastases rather than causing the transformation of existing lymphoid tissues to neoplastic cells.

There was moderate proliferation of lymphoid tissue, particularly in the liver, but no more than may occur in various infectious diseases of the bird.

Gey *et al.*[8] have reported that certain mouse tumors could withstand freezing at dry ice temperatures and, upon subsequent thawing and transplantation into susceptible mice, would produce tumors. In their experiments, lyophilization of the tissue also gave positive results. As a result of these experiments, they felt that they had demonstrated a virus as the causative agent of the tumor, since freezing at these temperatures would destroy the viability of cells.

From experience with this lymphoid tumor in fowls in which freezing to —78 C. (dry ice) does not prevent subsequent inoculation "takes" within two weeks, it is obvious that the tumor cells were not destroyed by the freezing process. The latent period of its cell-free agent is 100 to 150 days; however, from cell grafts tumors arise in less than two weeks. The results of these findings on survival of frozen tumor material in addition to those previously reported by Burmester *et al.*[5] and Breedis and Furth[9] indicate that tumor cells can survive at these temperatures.

SUMMARY AND CONCLUSIONS

The progressive development at the site of implantation, the later evolution and the terminal spread of a malignant lymphoid tumor (RPL No. 16) in chickens showed striking uniformity in birds inbred for both resistance and susceptibility to the spontaneous disease, lymphomatosis.

Metastatic tumor cells were seen in the visceral organs of both resistant and susceptible lines. The tumor spread was apparently associated with necrosis of the blood vessels and subsequent entrance of tumor cells into the circulation. Once in the circulation, there was a rapid accumulation of tumor cells in the liver sinusoids, kidney, and proventriculus, causing hemorrhage and necrosis, and leading to death a day or two later.

There was no indication that the general lymphoid tissues of the body became neoplastic. There was a systemic proliferative response similar to that seen in certain other infectious diseases of the fowl. This response was most marked in the resistant birds.

The results of the experiment indicate that the cellular implant has the ability to overcome inherited resistance to naturally occurring lymphomatosis.

The tumor specimen used in the experiment had been frozen at solid CO_2 temperature for sixteen months prior to use. Upon inoculation into the breast muscle of week-old chicks, tumors arose at the site of the implant within two weeks. Since the latent period of fowl lymphomatosis following injection of the filterable agent is more than three months, it is concluded that the tumor cells from the frozen sample were viable.

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Effect of Transplantable Lymphomatosis (Strain RPL 16) on Young, Rapidly Growing Chickens

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A SIGNIFICANT ASPECT of neoplastic growth is the effects of a tumor cell population on its host. The effects of a particular strain of transplantable avian lymphomatosis on a group of hosts has been evaluated primarily from the aspects of the percentage of inoculated birds which succumb to the tumor cells and the "latent period" or time intervening between inoculation and death of the hosts. Gross and microscopic pathology, hematological changes, and changes in body temperature have been reported for a variety of the transplantable strains of avian lymphomatosis.[1-3] In work dealing with RPL 16 and other tumors, Burmester and Prickett[1] report that results of examinations of the circulating blood of chickens inoculated with cell suspensions of the tumor were variable. Among the characteristics observed were increase in total white cell count and decrease in lymphocytes. These findings emphasize the extravascular characteristics of many of the transplantable strains of lymphomatosis. Usually, such observations have been secondary to other studies. Little attention seems to have been paid to such features as the effect of the tumor cells on the total growth of the host or on the growth of individual organs. Such features are of interest in gaining a better understanding of the pathology produced by transplantable strains of avian leukosis. The purpose of this paper is to report studies of the effect of a transplantable strain of visceral lymphomatosis (RPL 16) on the growth of young chickens, on growth of selected organs, and on changes of red and white blood cell levels and of body temperature.

METHODS

Strain RPL 16 was obtained through the courtesy of B. R. Burmester of the Regional Poultry Laboratory at East Lansing, Mich. Cellular material from the thirty-sixth serial passage was transported to the University of California in the fall of 1947. Serial cellular transplants were continued until the summer of 1949. Some 70 serial and side passages were conducted while the tumor was under study.

The strain was isolated by Burmester and Pricket.[1] It has received considerable attention from other workers.[2, 4-9] It has been demonstrated to grow satisfactorily in chicken embryos, in young, rapidly growing chickens, and in adult chickens.[1, 6, 10] It is characterized by the production of local tumors at the site of inoculation and by a high frequency of involvement of abdominal organs, resulting in death of a large percentage of inoculated birds. Experience with RPL 16 indicates that the pathology produced, and to some extent the latent period, depend upon the route of administration of the tumor cells. Intramuscular inoculation into the muscles of the posterior aspect of the tibial segment of the limb was the route of choice for most studies conducted with RPL 16 in California. Such inoculation in young birds produced local enlargement of the leg, enlargement of the liver and spleen, and, usually, enlargement of the kidneys. The results presented below were derived from two experiments dealing with transplanted cells of RPL 16 in young, single-combed White Leghorn chickens.

Experiment 1.—The first experiment was designed to determine the change of weights of liver, spleen, and kidney of young, rapidly growing normal and inoculated birds. Forty-seven birds of approximately 3 weeks of age were inoculated in the leg muscles with 0.5 cc. of a suspension of tumor tissue in Ringer's solution. The inoculum was taken from the twenty-fourth serial passage of RPL 16 in California. Seventeen control chicks were handled in the same manner as the experi-

TABLE 1—Average Weights in Grams of Organs of Normal Chickens and Chickens Inoculated with a Strain of Transplantable Lymphomatosis

LEUKOTIC					NORMAL				
TIME IN HOURS	NO. OF BIRDS	AVERAGE WEIGHT OF ORGANS			TIME IN HOURS	NO. OF BIRDS	AVERAGE WEIGHT OF ORGANS		
		LIVER	SPLEEN	KIDNEY			LIVER	SPLEEN	KIDNEY
102	6	3.75	0.14	0.54	102	4	4.06	0.72	0.60
124	6	6.42	0.28	0.81	124	3	7.02	0.24	0.84
148	6	6.86	0.29	0.83	148	2	7.11	0.35	1.23
168	6	8.94	0.21	1.09	168	2	6.08	0.29	0.80
190	5	8.40	0.38	1.04	190	2	6.54	0.16	0.92
200	5	9.64	0.30	1.17	200	2	8.60	0.31	1.24
216	2	11.30	0.53	1.64	216	2	7.76	0.30	1.06

From the School of Veterinary Medicine, University of California, Davis.

mental birds but did not receive the tumor cells. The average weight of the experimental birds was 112 Gm. (range 88 to 128) at the time of inoculation. The average of the controls was 149 Gm. (range 132 to 161). Twelve of the inoculated chicks died at an average of eight days after receiving the tumor cells. The times of death of these birds are presented in the form of a superimposed histiogram in figures 2, 3, and 4. Four days after inoculation, groups of leukotic and control birds were autopsied. The liver, spleen, and the kidney from the side corresponding to the site of injection were weighed. The results of these determinations are presented in table 1.

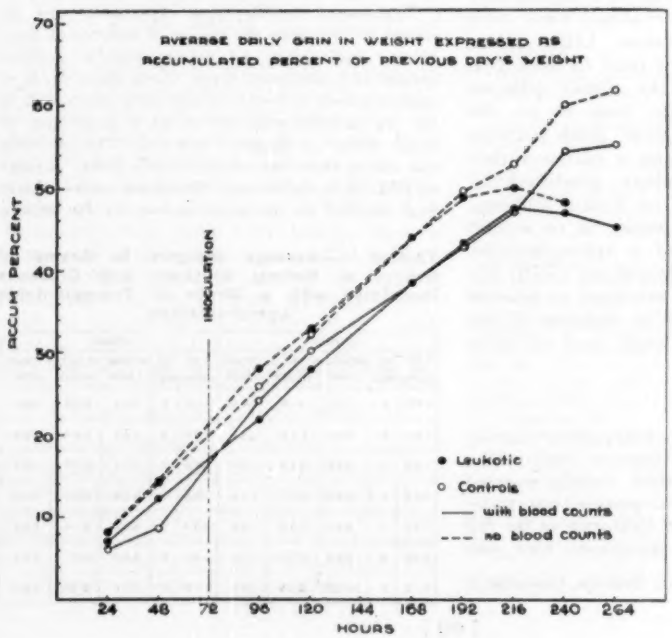
Experiment 2.—The second experiment was designed to determine the effect of the tumor upon the growth of young chickens, upon the body temperature, and upon circulating red and white cells. Twenty-two, 26-day-old birds of an average body weight of 115 Gm. were studied. The birds were divided into two groups; one received tumor cells, the other acted as a control group. Four birds from each group were studied daily in regards to their red blood cell levels, total and differential white blood cell levels, and body temperature. All birds were weighed daily. The birds were inoculated, in a manner similar to those of the first experiment, with tumor cells of the thirty-first serial passage of the tumor. The average time of death of the inoculated birds was eight days, indicating that the tumor at this stage was comparable to the tumor activity at the time of the twenty-fourth passage.

Techniques For Blood Cell Counts.—Shaw's technique was utilized for the determination of total red cells and total white cells.[31] This method uses a stained diluting fluid and permits red cells and white cells to be counted in one dilution and by charging the counting chamber but once. Differential white cell counts were conducted by employing supravital staining techniques as outlined by McClung.[17] Twisselmann [32] has shown the supravital technique to be more satisfactory than stained preparations. Blood studies were initiated three days before inoculation.

RESULTS

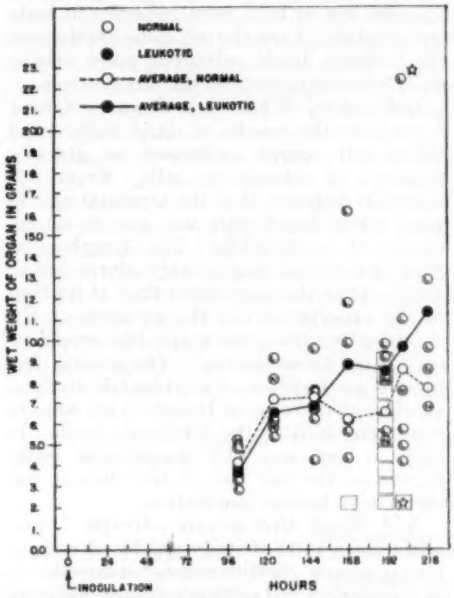
Effect on Body Weight.—Graph 1 demonstrates the increase in body weight of leukotic and normal birds expressed as accumulated percentage of the previous day's weight. The birds, which were subjected to daily blood examinations, were plotted separately as it was found that the restriction of diet during the daily four-hour examination period influenced the weight gains. Leukotic birds grew at about the same rate as the normal birds until forty-eight hours before death when the gains decreased or ceased and the birds actually lost weight.

Effect on the Size of the Liver.—Data pertaining to the leukotic and normal birds



Graph 1—The effect of transplantable lymphomatosis on the growth of young chickens. Inoculated birds grew at approximately the same rate as controls until forty-eight hours before death, when growth ceased. The weights of birds which were subjected to daily blood studies are plotted separately.

destroyed in order to follow changes in organ sizes are presented in table 1. Information on the liver is presented in graph 2. The average weights of the livers

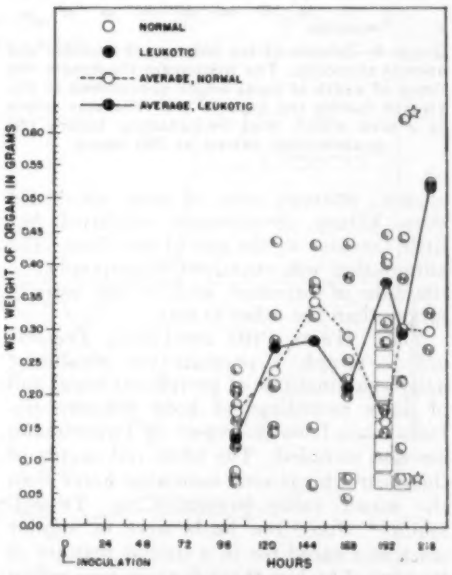


Graph 2—Growth of the livers of leukotic and normal chickens. The histogram illustrates the times of death of birds which succumbed to the disease during the course of the experiment. The star refers to a bird which died immediately before the examination period at 200 hours.

of leukotic birds increased noticeably at about the seventh day after inoculation; however, observation of the individual weights of the experimental animals reveals that some of the livers were apparently enlarged as early as five days after inoculation. These cases of early enlargement of the liver may have represented cases that would have died early if the disease had been permitted to take its natural course. In contrast, some of the livers seemed not to have been involved even after the greater number of unexamined birds had succumbed to the disease. These features emphasize the rather wide variation of incubation periods which may be encountered in a single passage of transplantable leukosis.

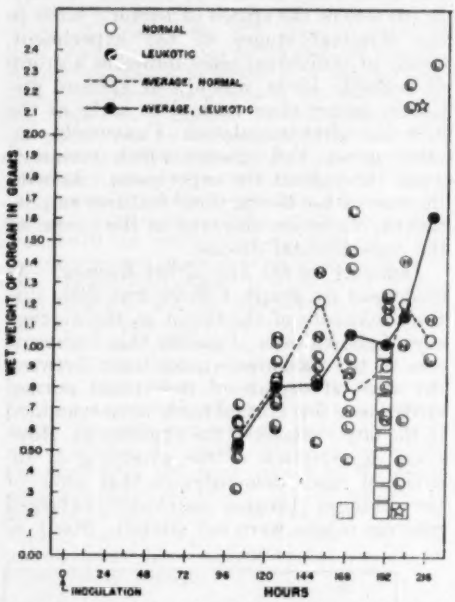
Influence on the Size of the Spleen.—In the chicken, the spleen is relatively small as compared to man. It is one of the more variable organs of the body, especially in young birds. Graph 3 indicates that there was a tendency toward an average increase in the size of the spleen of leukotic birds in the terminal stages of the experiment. Study of individual cases indicates a group of leukotic birds which had spleens distinctly larger than normal as early as the fifth day after inoculation. Conversely, another group had spleens which remained small throughout the experiment. As with the case of the livers, these features emphasize the variation observed in the course of the experimental disease.

Influence on the Size of the Kidney.—As evidenced by graph 4 there was little distinct influence of the tumor on the average size of the kidneys. Possibly this feature is due to the variation encountered between the sizes of organs of individual normal birds, since few normal birds were examined in the latter phases of the experiment. However, examination of the grouping of individual cases demonstrates that some of the kidneys became markedly enlarged whereas others were not altered. Study of



Graph 3—Growth of the spleens of leukotic and normal chickens. The star refers to a bird which died immediately before the examination period at 200 hours.

individual cases indicates that some of those birds which did not exhibit enlargement of the kidney also did not exhibit detectable gross enlargement of other visceral



Graph 4—Growth of the kidneys of leukotic and normal chickens. The histogram illustrates the times of death of birds which succumbed to the disease during the experiment. The star refers to a bird which died immediately before the examination period at 200 hours.

organs; whereas, some of those which did show kidney involvement exhibited but little increase in the size of the liver. The kidney that was examined corresponded to the side of injection and it was usually larger than the other kidney.

Total White Cells and Body Temperature.—Graph 5 presents the results of daily examinations of peripheral blood and of daily recordings of body temperature. Data taken from the report of Twisselmann are also included. The white cell counts of the control birds were somewhat lower than the mean value presented by Twisselmann.[13] Since the birds were of similar stock and cared for in a similar manner as those used by him the difference may reflect an age difference of birds used in the two studies. The total white cell levels in the inoculated birds were more variable than the controls. During the last forty-eight hours of life, a distinct rise in total white cells occurred. This prominent terminal rise in leukocytes was noted in 3 of the 4 leukotic birds. The shift in leukocyte count appears not to have been reflected in body temperature. Considering individual cases, the leukotic birds exhibited more erratic daily temperatures than did the controls.

Differential White Cell Counts.—Graph 6 presents the results of daily differential white cell counts expressed as absolute numbers of circulating cells. From this figure, it appears that the terminal rise in total white blood cells was due to an increase of neutrophils. The lymphocytes were not raised appreciably above initial levels. It is also significant that at no time in the experiment did the numbers of circulating lymphocytes reach the levels recorded by Twisselmann.[13] The general feature of an increase of neutrophils with no significant increase of lymphocytes was encountered in 3 of the 4 leukotic birds. In only 1 bird was the lymphocyte count higher on the last day of life than it was three days before inoculation.

Red Blood Cell Levels.—Graph 7 presents the results of daily red blood cell determinations. No differences between levels of circulating red cells before and after inoculation with the tumor were found that could not be accounted for by the natural variation of blood cell levels in the chicken. The red cell values were lower than those reported by Twisselmann. Again, this may have been a reflection of the differences in age of the birds (1.5 to 8.5 months of age as compared to 3-week-old birds used in this work).

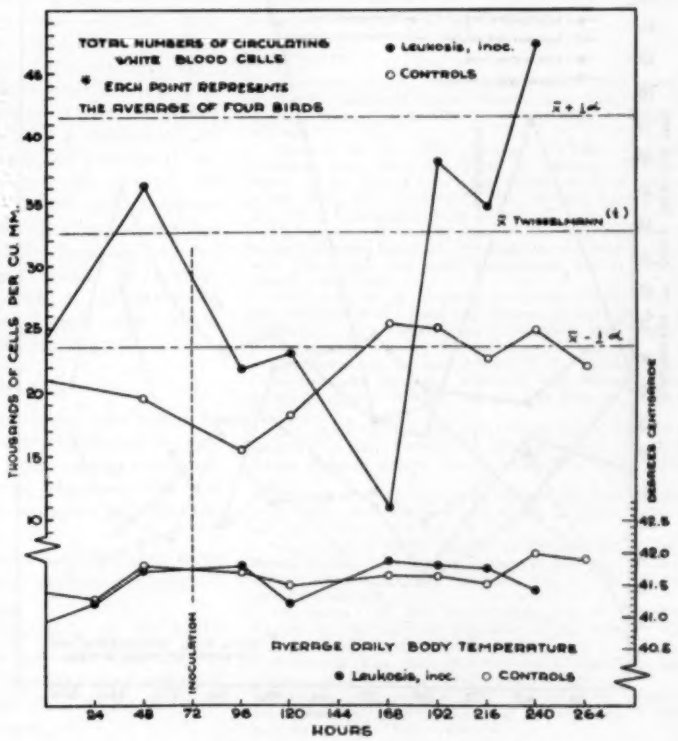
DISCUSSION

The data presented by graphs 1, 3, 4, and table 1 reveal that whereas total body growth was essentially at a standstill in the last forty-eight hours of the experimental disease, the internal organs such as the liver and spleen and, sometimes, the kidney were enlarged greatly. In some animals, there was a twofold increase in the tissue mass represented by the kidney, liver, and spleen as compared to normal birds of comparable body weight. A possible reason for the cessation of total body growth is the interference with the synthetic activity of the liver or the vital functions of some organ which apparently occurred about seventy-two to forty-eight hours be-

fore death. The fact that total body growth ceased whereas the tumor proceeded to increase in the liver, spleen, and kidney strongly suggested that tissue substances of the animal itself are utilized by the tumor in terminal stages of the disease. Similar observations have been made in the study of transplantable mammalian neoplasms.[14] In considering organ weights of individual cases, it appears that those birds which showed enlarged livers also had enlarged kidneys. This feature was consistent whether it appeared early in the period of observation or after the peak of the disease process, and may indicate the influence of the time of onset of metastases from the original site of tumor growth. The pathology produced by the tumor in individual cases and the time of death might be directly correlated with the time at which metastasis occurs and/or the ability of the tumor cells to increase in the secondarily involved organs.

Both from evidence presented by the above experiment and preliminary histological studies, it appears that visceral organs become noticeably involved by the tumor at, or shortly after, the midpoint of the latent period. On the basis of histological evidence of destruction of veins in the vicinity of the local tumor growth it might be assumed that the visceral organs become involved by the metastasis of tumor cells; however, there is a possibility that tumor cells enter the viscera early in the experimental period but do not multiply sufficiently to be detected grossly or by random sectioning until the midpoint of the latent period. Experiments using tagged tumor cells might prove to be profitable in clarifying this question. Such a possibility is currently under study. Evidence that enlargement was due to massive metastasis rather than leukemic infiltration is apparent from the fact that the number of lymphocytes of the circulating blood was not elevated.

In the experiments described above, it was found that in many cases kidney involvement occurred on the side of the body

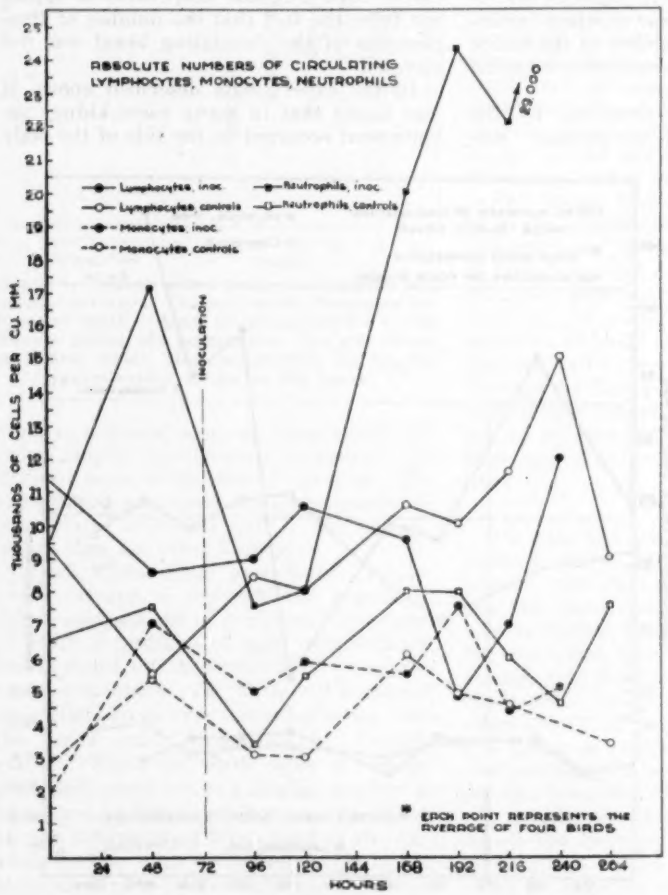


Graph 5—Effect of transplantable lymphomatosis on the total white blood cell levels and body temperatures* of chickens. There is a distinct terminal increase in circulating white cells (t). The mean and range including one value of standard deviation as established by Twisselmann[15] is shown for comparison. No significant alteration occurred in body temperature of the birds.

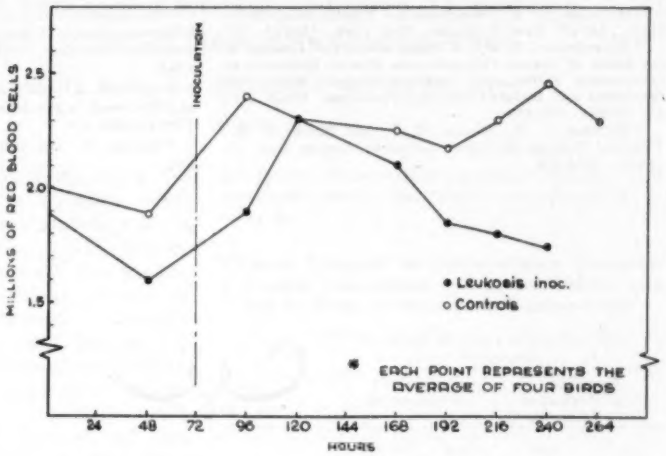
into which the inoculation was made. Thus, when the right leg was the site of injection, the right kidney usually showed more involvement by the tumor than did the left kidney. If hematogenous transfer of the tumor cells is assumed to be the major mode of metastasis of RPL 16, the above observation may constitute evidence for the presence of a functional renal portal system in the kidney of the domestic chicken.[15, 16] The kidney of the side corresponding to the site of injection presumably was exposed to more tumor cells being freed from the site of local growth.

It should be emphasized that in an experiment of this type, in which animals are destroyed at intervals beyond the average lethal period of the disease, the disease picture is conditioned by the lethal period so that later samples do not give an average picture of the influence of the tumor on the various organs. This point is emphasized by the weights of the organs of 1 bird which died just before the examination period and thus was included in the group that was examined at 200 hours. The weights of the organs of that bird have been marked by a star in graphs 2, 3, and 4. In each case the organ was very large.

In the above experiments, it was seen that the increase in total white blood cells was due primarily to an increase in neutrophils. This increase began at about the time of invasion of the parenchymatous organs by leukotic cells (graph 6). This feature may suggest that the increase in



Graph 6—The effect of transplantable lymphomatosis on the circulating white cells of the chicken. There is a terminal increase in neutrophils.* The final levels of lymphocytes are not appreciably higher than preinoculation levels.



Graph 7—The effect of transplantable avian lymphomatosis upon circulating red blood cells.* No clearly defined alteration can be seen during the seven-day latent period.

neutrophils was elicited by products of tissue destruction brought about by the invasion of the organs by tumor cells.

There was no significant alteration in the red blood cell levels of normal and leukotic chicks. The final difference in red blood cell levels between leukotic and normal birds was but twice the variation seen before inoculation. It is probable that a disease of such a short course (seven to eight days) would not greatly influence the red blood cell levels, unless it caused active degeneration of the cells or produced extensive hemorrhages, since the life of the nucleated chicken red blood cell is about twenty-eight days.[17] Evaluation of any influence of transplantable leukosis on red blood cell levels might better be accomplished by a technique which evaluates the formation and release of new cells rather than the survival of circulating cells.

SUMMARY

1) Two experiments were conducted to determine the effect of transplantable lymphomatosis (RPL 16) upon young chickens.

2) The growth of the chickens was not altered until the last quarter of the experimental period.

3) The livers, spleens, and kidneys were enlarged. Considerable variation occurred among individual cases.

4) The kidney of the side of the body corresponding to the site of inoculation was always larger. This fact may support the assumption that the chicken has a functional renal portal system.

5) A definite increase occurred in the white cell levels of leukotic birds. Differential studies showed the increase to be due to neutrophils. Levels of red cells, lymphocytes, and other white cell types were not markedly altered. No significant changes occurred in body temperature.

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The Speed of Immunity Response Following Vaccination with the B_1 Strain of Newcastle Disease Virus

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VACCINATION of day-old chicks with the B_1 strain of Newcastle disease virus (NDV)[1,2] causes only a transient rise in hemagglutination-inhibition (HI) titers, but chicks thus vaccinated are resistant to exposure with fully virulent strains of NDV.[1-6] It has been suggested [1,3] that this resistance may result in part from a cell block or interference phenomenon occurring in the susceptible respiratory epithelium. It was observed [1] that birds vaccinated intranasally at 39 days of age were resistant to intranasal challenge with NDV strain 11914 within twenty-four hours after vaccination but that chicks vaccinated at 1 and 16 days of age did not resist challenge until four and six days postvaccination, respectively. These findings were interpreted as indicating an earlier antibody response in the older birds similar to the findings of Wolfe and Dilks.[7]

The present study was undertaken to determine how early immunity against two routes of challenge exposure develops in chicks following vaccination with the B_1 strain of NDV at 1 day of age.

PROCEDURES

Experiment 1.—A total of 104 day-old White Rock chicks from NDV-susceptible flocks were vaccinated ocularly [8] with one drop of vaccine commercially prepared by freeze-drying methods. The titer of the reconstituted vaccine was $10^{6.2}$ embryo lethal dose $_{50}$ (e.l.d.$_{50}$) per milliliter, as assayed in 10-day-old embryonating hens' eggs from NDV-susceptible flocks.[8] A like number of nonvaccinated hatch mates were held in isolation as controls. At twenty-four hours postvaccination and thereafter on each of ten succeeding days, 5 vaccinated chicks and 5 controls were removed to separate quarters and challenged ocularly with approximately 10^7 e.l.d.$_{50}$ of NDV strain 11914. At the same intervals, 5 vaccinated chicks and 5 controls were exposed to the same dose contained in 0.1 ml. injected into the thigh muscle. The chicks were individually identified by wing bands. The criterion of protection during the four-week postchallenge observation period was survival with the absence of any paralytic or noticeable respiratory symptoms. For purposes of tabulation, chicks exhibiting such symptoms were considered dead. The results are presented in table 1.

TABLE 1—Onset of Demonstrable Immunity Following Vaccination of Day-Old Chicks with the B_1 Strain of Newcastle Disease Virus

Challenge: days post-vaccination	No. chicks dead in 4 weeks/No. chicks challenged			
	Challenged intraocularly		Challenged intramuscularly	
	Vaccinated	Controls	Vaccinated	Controls
1	5/5	5/5	5/5	5/5
2	0/5	5/5	5/5	5/5
3	0/5	4/5	5/5	5/5
4	0/5	4/5	5/5	5/5
6	0/5	4/5	3/5	4/5
7	0/5	5/5	1/5	5/5
8	0/5	5/5	0/5	5/5
9	0/5	5/5	0/5	5/5
10	0/5	5/5	0/5	5/5

Experiment 2.—Having observed a marked difference in the resistance to ocular and intramuscular challenge at various time intervals, it was decided to repeat the experiment using larger numbers of chicks and limiting the challenges to their apparently "critical" times. From the same source as before, 200 day-old White Rock chicks were vaccinated ocularly with on drop of reconstituted commercial B_1 vaccine which titered $10^{6.25}$ e.l.d.$_{50}$ per milliliter. One hundred hatch mates were held in isolation as nonvaccinated controls. At twenty-four and forty-eight hours postvaccination, 50 vaccinated chicks and 25 controls were challenged ocularly with 10^5 e.l.d.$_{50}$ of NDV strain 11914. At seven and eight days postvaccination, a like number of vaccinated chicks and controls were challenged with 10^6 e.l.d.$_{50}$ of NDV 11914 contained in 0.1 ml. injected into the thigh muscle. The birds were observed over a three-week period postchallenge, again using survival with the absence of respiratory or paralytic symptoms as the criterion of protection. The results are given in table 2.

DISCUSSION

If the resistance to challenge exposure following vaccination with the B_1 strain of NDV were due solely to the formation of circulating neutralizing antibodies, one would expect to have found little or no differences in the time intervals after vaccination required to protect against either

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the respiratory or intramuscular routes of challenge. A significant difference was observed, however. The results thus indicate

TABLE 2—The Susceptibility of Chicks to Two Routes of Challenge Exposure at Various Time Intervals Postvaccination

Interval between vaccination and challenge	Route of challenge	No. chicks dead in 3 weeks/ No. chicks exposed			
		Vaccinated	Per cent	Controls	Per cent
24 hours	O	25/50	50	25/25	100
48 hours	O	4/49*	8	25/25	100
7 days	IM	28/50	56	25/25	100
8 days	IM	9/46	19	25/25	100

* One chick died on the first day postchallenge; O—Ocular; IM—Intramuscular.

that the immunity engendered by intranasal or ocular vaccination with the B_1 strain is the result of at least two immunological mechanisms. One, a local immunity in the respiratory epithelium which is demonstrable within the first forty-eight hours postvaccination, could be either the cell block phenomenon previously suggested [1, 8] or could be the action of noncirculating neutralizing antibodies produced locally by infected cells of the respiratory tract. The second, apparent at about the eighth day postvaccination, most probably is the formation of effective levels of neutralizing antibodies circulating systemically. The probability that local immunity was responsible for the observed resistance to intramuscular challenge after the eighth day is minimized in view of the known low invasiveness of the B_1 strain for tissues other than those of the respiratory tract.[9] It is known that neutralizing antibodies are formed in response to B_1 vaccination,[10] although this is not the general belief because most serological assays reported in the literature are based upon HI tests rather than on the more expensive and time-consuming serum-neutralization (SN) tests. It has been shown,[10, 11] however, that the HI test is not an accurate measure of immunity to Newcastle disease. Birds may show insignificant HI titers in their serums and still be refractory to challenge exposure. Such birds invariably have significant SN titers, however.[10, 11]

Our results were at variance with those of Hitchner and Johnson.[1] This may be explained by comparing the relative severity of challenge exposures. They[1] challenged their birds with 0.05 ml. of undiluted allantoic fluid from embryos infected with NDV strain 11914. In our first experiment, the challenge exposure was less than 0.01 as severe, though the challenge dose killed or paralyzed 80 to 100 per cent of the control birds each time. The exposure used in our second experiment was tenfold more severe than in the first experiment but still represents only 0.1 of the dose of Hitchner and Johnson. It is highly improbable that natural exposure under field conditions would present as great a test of immunity as the doses used in our experiments, judging from the observations of Doll *et al.*[10] The challenge doses we employed represented approximately 100 and 1,000 lethal doses in 3-week-old chicks, according to observations [12] made in this laboratory.

Another probable explanation for the disparity between our results and those of Hitchner and Johnson[1] is related to the respective virus titers of the vaccines used in the experiments. Their vaccines titered 10^5 and 10^6 per 0.1 ml., as compared to titers of $10^{6.32}$ and $10^{6.35}$ per milliliter used in the present study. This difference becomes significant [13] especially if a cell block mechanism is the primary factor in rapid immunity.

SUMMARY

Immunity against respiratory exposure with a virulent strain of NDV was demonstrable within forty-eight hours following ocular vaccination of day-old chicks with the B_1 strain of Newcastle disease virus. Resistance to intramuscular challenge was not found until approximately the eighth day postvaccination. These findings suggest that a cell block phenomenon results from the vaccination, followed by the formation of specific neutralizing antibodies in the circulatory system.

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Avian Spirochetosis in California Turkeys

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SINCE there have been several outbreaks of avian spirochetosis reported in turkeys in California during the past few years, there is recognized a particular need for investigating the transmission of this infectious disease. This is true in view of the economic bearing the disease has to owners of turkey farms, since means of preventing spread of the infection are not available at present. It is known that the fowl tick or "blue bug," *Argas persicus* (Oken) 1818, is a vector of avian spirochetosis in some regions and therefore the purpose of the study presented herein was to determine whether this tick could be incriminated in the transmission of spirochetosis occurring in turkeys in California.

HISTORICAL

The causative agent of avian spirochetosis, *Borrelia anserina* (= *Spirochaeta anserina*), was discovered by Sacheroff in 1891, while Marchoux and Salimbeni, in 1903, found the important method of transmission to be by the common fowl tick, *Argas persicus*. During the intervening years, many aspects of both the causative agent and the tick vector were exhaustively studied. Levaditi contributed to the knowledge of the pathology in the avian host. Such men as Nuttall, Prowazek, Swellengrabel, Balfour, Hindle, and Brumpt developed basic information on spirochetal taxonomy, vector and disease distribution, serology, host probabilities, and the life cycle of the spirochete in the definitive as well as in the intermediate host. It would, therefore, appear that little information could be added to what has already been confirmed.[9]

Spirochetosis in domestic fowl (ducks, geese, chickens, turkeys) and its transmission by *A. persicus* has been established for most of Continental Europe, Africa, India, Australia, Java, and South America but, until recent years, was not recorded from North America. The presence of this disease in the United States was suggested as early as 1917 when outbreaks of paralysis in Texas chickens were reported by Kaupp.[1] Further outbreaks were reported by Ward and Gallager,[2] but all three men failed to mention any positive diagnosis. Since the known tick vector occurs in South America, Panama, Mexico, the southwestern United States, and as far north as Canada, one should not be too surprised to find *B. anserina* in domestic poultry of North America. Thus in 1941, Brown and Cross[3] reported their results from a year's study on what appeared to be avian spirochetosis in Texas and the successful transmission by *A. persicus*. During a three-year period, 1937 to 1939, mortality from so-called "range paralysis" ranged from 27 to 91 per cent in chickens at the Texas College of Arts and Industries. Exposure of normal poults to *A. persicus* taken from pens housing paralyzed birds resulted in transmission of a fowl paralysis. Although their numerous experiments suggest that the ticks were involved in the transmission of the paralysis, no evidence was presented as to the nature of the causative organism. Burroughs[4] reported in 1947 the successful transmission of avian spirochetosis by *A. persicus* which had been obtained from a poultry raiser in El Paso, Texas. Larvae and nymphs of the original collection of ticks proved infective for normal White Leghorn chickens. Blood smears showed spirochetes of the *anserina* type and the clinical manifestations were similar to those of avian spirochetosis.

CALIFORNIA PICTURE

An outbreak during the winter of 1945 was restricted to turkeys raised in one area of the northern San Joaquin Valley. Hoffman, Jackson, and Rucker,[5] and Hoffman and Jackson[6] made a study of this outbreak and found that the causative agent presented a clinical and pathological picture similar to that caused by *Borrelia anserina*. The surprising factor in this outbreak was the absence of the tick vector on the turkey farm where the diseased birds were housed, and although a thorough study of the epizoötiological factors was made relating to this outbreak, no explanation was offered as to the method of transmission.

A second outbreak of this disease occurred in 1947 and likewise was found to appear in turkeys during the winter months. McNeil, Hinshaw, and Kissling[7] were confronted with findings similar to those of Hoffman *et al.*[5] in 1945. McNeil and her co-workers, however, proved that bile droppings from infected birds were infective to normal poults upon ingestion, and that this method of

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transmission could well play an important part in the dissemination of the infection. From a direct quotation in McNeil's paper, "No ticks, lice, nor mites were present at any time," the important difference is obvious in the epizoötiology of these two outbreaks in California.

A third outbreak in turkeys occurred during February, 1950, in the central San Joaquin Valley. The diseased birds were examined both clinically and pathologically by veterinarians of the California State Poultry Pathology Laboratory. Spirochetes isolated from the blood and organs of the birds submitted to the laboratory proved morphologically identical to *B. anserina*. Investigation of the turkey farm resulted in the collection of large numbers of *A. persicus*. These ticks were found with equal ease in the turkey shades, feed boxes, and the houses to which the diseased birds had access. Unfortunately, the entire flock was sold before birds could be obtained for transmission tests. Nevertheless, a sample of ticks was returned to the University of California at Berkeley where transmission tests were conducted.

Experimental Methods

In an attempt to transmit the organisms by feeding ticks on clean birds, approximately 900 ticks collected from the turkey farm were segregated, according to sex, stage of nymphal development, and state of engorgement, into ten lots for feeding tests. These ten lots were kept at constant temperatures for different periods of exposure prior to feeding as indicated in table 1. This procedure was adapted after the work of Borrel and Marchoux.[8] According to these workers and subsequent investigators, an extreme spirochetosis developed within the ticks at 35 C., even though the ticks were kept at room temperature for the previous three months.

In an attempt to transmit infection by inoculation of triturated tick tissue, eight tests were carried out on the ticks which had fed on uninfected birds. The ticks were kept at constant temperatures for varying periods after feeding and before trituration as indicated in table 1. This procedure was undertaken with engorged specimens inasmuch as Knowles, DasGupta, and Basu[9] had found that feeding on a normal bird would hasten the development of the spirochete cycle in the tick. The ticks were triturated in a mortar with 0.5 Gm. of sterile sand and 2 cc. of sterile normal saline. The triturated material was centrifuged for two minutes at 1,000 r.p.m. Throughout the whole procedure, the material was maintained at temperatures equal to the respective lot of ticks to be triturated. Smears were made from both the supernatant fluid and the residue and stained with giemsa and gentian violet. One cubic centimeter was inoculated intramuscularly into the right gastrocnemius of each bird tested.

In two *per os* inoculation tests, intact ticks were forced down the throat of each bird. The ticks were kept at constant temperatures for different periods of time after allowing them to feed and before *per os* inoculations as indicated in table 1.

A relative humidity of 75 plus or minus 5 per cent was maintained in the temperature cabinets in which the ticks were stored before all tests.

In submitting birds to feeding by Argas, the legs of each bird were tied together and a muslin cap was tied over the head. This precaution prevented the birds from eating the ticks. The birds were laid on a table with the wings spread flat and the ticks were shaken from the holding vials directly on the body below the wings. The birds were transferred to the bottom of a large crock which had been previously lined with sheets of paper to absorb the fecal discharges. The tops of the crocks were covered with black muslin cloth to reduce the quantity of light and in turn to reduce the activity of the bird. Throughout the entire study all birds were kept in individual wire cages which were constructed so that the feces would drop through the bottom wire netting. At no time were any of the birds afforded cohabita-

TABLE 1—Transmission Tests of Borrelia Anserina Among Chickens and Turkeys with Argas Persicus

Feeding				*Per os inoculation*				Intramuscular inoculation			
	Condition of ticks pre-feeding				Condition of ticks pre-inoculation				Condition of ticks pre-inoculation		
Test No.	temp. C.	days	No. ticks fed	Test No.	temp. C.	days	No. ticks inoc.	Test No.	temp. C.	days	No. ticks inoc.
1	24	14	39	1A	24	2	39				
2	32	5	72					2A	35	4	72
3	34	7	74					3A	30	7	74
4	28	5	65					4A	28	5	65
5	30	5	66					5A	30	7	48
6	34	4	109					6A	35	4	58
7	40	7	88					7A	40	7	76
8	41	7	32	8A	41	7	32	8B		*	81*
9	24	28	38					910A		**	59
10	40	5	21								

* Surplus from feeding tests 5, 6, 7, and triturated immediately postfeeding. ** Triturated immediately postfeeding.

tion. The birds were fed on a high protein diet and with the exception of the baby chickens were kept outdoors.

Daily blood smears were taken from birds for two days before each test and every twenty-four hours thereafter up to a ten-day period. The blood smears were also stained with giemsa and gentian violet.

RESULTS

Of over 800 ticks given the opportunity to feed, only 604 engorged. Of these 604 ticks, 185 fed on a total of 5 White Leghorn chickens (6 weeks to 2 months of age), while 360 ticks fed on a total of 5 turkeys (1 to 2 months of age). The remaining 59 ticks fed on 12, 3-day-old White Leghorn chicks. There was not the slightest evidence of any signs of avian spirochetosis in the birds on which the ticks fed and all blood smears were negative. One turkey died on the third day postfeeding with symptoms of diarrhea, anorexia, and slight paralysis of the legs before death. Upon postmortem examination, all internal organs were normal as was the blood picture immediately before and after death.

Five hundred and thirty-three engorged ticks were triturated for the intramuscular inoculation tests. The inoculations were made into a total of 8 birds (3 chickens, 6 and 8 weeks old; and 5 turkeys, 4 to 8 weeks in age). These birds were likewise negative for spirochetosis throughout a ten-day postinoculation period. Smears made from both the supernatant fluid and residue of each lot of triturated ticks were also negative for spirochetes.

A total of 71 engorged whole ticks were administered *per os* to 2 birds (39 to a 6-week-old chicken and 32 to a 10-week-old turkey) with negative results.

DISCUSSION

It seems very unlikely from the results that any of the ticks harbored spirochetes or were involved in the transmission of spirochetosis to the turkeys on the infected premises. It is certain that the majority of the ticks collected on the farm had fed on the turkeys because there were no other fowl on the premises to supply blood meals.

Although the percentage of ticks found to be infective in nature varies, information from the laboratory experiments of Knowles, DasGupta, and Basu [9] show that ticks collected in the field, which were fed on infected birds in the laboratory, 40 per cent of the males and 60 per cent of the females proved infective at three months after the feeding period. In spite of the large number of specimens collected in the 1950 California outbreak, no ticks were found infective in the current tests reported. Furthermore, *B. anserina* apparently was not harbored in the ticks since no positive infections were established in normal birds following inoculation of tick tissues by the various routes used. Contrary to these findings are the experiments of Knowles, DasGupta, and Basu [9] in which they obtained 100 per cent transmission by intramuscular inoculations and 53 per cent transmission by *per os* inoculations of infective *A. persicus* suspensions.

An insufficient number of birds may have been used in the individual feeding trials (2 per test). This may be true in view of previous tests conducted by Knowles, DasGupta, and Basu.[9] These workers obtained a 44 per cent rate of transmission upon feeding 600 ticks on 29 birds.

The 1 turkey (age, 4 weeks) which died after completion of the second feeding test probably succumbed as a direct result of the feeding of the ticks. From it, 65 replete nymphs were recovered.

The negative results, coupled with the discoveries made by the investigators of the 1945 and 1946 outbreaks in California, suggest another mode of transmission; that is, the possibility of diseased birds having been introduced to the main flock and transmission by ingestion of infective feces. The absence of *A. persicus* during the previous outbreaks in California tends to support this viewpoint, whereas, in the 1950 outbreak, the presence of these ticks may have been entirely incidental to the cycle of transmission. Sreenivasan and Sankaranarayan [10] claimed that ingestion of infective feces was the important method of transmission in an outbreak in India which failed to disclose the presence of a tick vector. The possibility of the involvement of another vector has not been investigated.

The pigeon tick, *Argas reflexus*, has occasionally been found to be infected naturally with *B. anserina* in other parts of the world, and although it somewhat parallels *A. persicus* in distribution, it has

never been found to occur or ineriminated in the outbreaks in North America. Fülleborn and Mayer [11] have shown that the only other tick which is capable of transmitting *B. anserina* is one which is native to Africa *(Ornithodoros moubata)*. This tick would therefore have no bearing on the transmission of the disease in North America.

The question of mite transmission has been studied through experimentation with the common red mite of poultry, *Dermanyssus gallinae*. After four years of study of avian spirochetosis, Knowles, DasGupta, and Basu [9] claimed that this mite may not serve as an efficient vector since there is no multiplication of spirochetes in the intestinal tract and the organisms are observed only up to three days after feeding. On the other hand, Hungerford and Hart [12] found the red mite in Australia to serve as a good vector, experimentally. Since these workers failed to take into consideration the possibilities of infection through cohabitation of their experimental birds, the work is open to question.

Mosquitoes have not been overlooked in the transmission cycle, for Zuelzer[13] claims she obtained positive transmission of *B. anserina* by Culex mosquitoes (species not named) in an outbreak occurring in a region of the Baltic Sea coast. *Argas* was not present in the chicken coops whereas the Culex mosquitoes were found to be abundant and infective for normal pullets. Also of interest in Zuelzer's report is the fact that this is the farthest north of the equator (54 to 60 degree parallels) that the disease has extended. A more detailed knowledge of the ability of mosquitoes to transmit spirochetosis comes from the experiments of Nieschulz and Bos [14] who discovered that *Culex pipiens*, *Aedes aegypti*, and *Culiseta annulata*, all may take in the spirochetes but that the organisms lose their pathogenicity in one to two days. Kapur [15] in India, worked with *Aedes albopictus*, and arrived at similar conclusions. All three workers are of the opinion that mosquitoes are of no practical importance in the transmission of avian spirochetosis.

Summary and Conclusions

The present status of avian spirochetosis with particular reference to California outbreaks has been reviewed.

Outbreaks in the United States and specifically in California are discussed.

Transmission of *B. anserina* was not obtained by any of the following routes with *A. persicus* taken from premises recently harboring infected turkeys: feeding, intramuscular inoculation of triturated ticks, and oesophageal introduction of whole ticks. Experimental birds included chickens and turkeys.

It is concluded on the basis of negative results with tick transmission trials, that infective bile droppings of turkeys represent the most probable means of transmission of avian spirochetosis in the reported California outbreaks.

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An Evaluation of Medicaments, with Special Reference to Teniatol for Removing Fringed Tapeworms (*Thysanosoma Actinoides*) from the Livers of Sheep

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LIVER condemnations due to the presence of fringed tapeworms (*Thysanosoma actinoides*) may run as high as 20 per cent of the annual lamb slaughter in Denver, according to Harshfield and Esplin.[5] These authors [5] reported a loss of 50,000 livers resulting from the presence of this parasite during a single year at one west coast slaughtering establishment. No percentages were given.

Because of the liver condemnations, together with the reputed deaths [1] resulting from the presence of fringed tapeworms, attempts have been made to develop a medicament that is capable of removing them from the liver and thereby reducing the losses.

It is the purpose of this paper to (1) present a summary of the various medicaments that have been employed by investigators in attempts to remove fringed tapeworms from the livers of sheep, (2) report the results obtained by us on feeder lambs medicated with teniatol,* and (3) evaluate on an economic basis the use of present-day thysanosomicides.

REVIEW OF LITERATURE

According to Christenson,[2] copper sulphate was recommended for and believed to be effective in removing fringed tapeworms from infected feedlot lambs in Minnesota, thereby reducing the high death rate that was occurring at that time.

Newsom and Cross [7] and Newsom and Thorp [8] stated that no effective treatment had been found for the removal of fringed tapeworms.

Harshfield and Esplin [6] reported that the administration of copper sulfate-nicotine sulfate repeated three to four times at monthly intervals is effective in reducing the percentage of livers condemned for fringed tapeworms. They found phenothiazine to be ineffective in removing the tapeworms.

Olsen and Allen [9] found carbon tetrachloride, tetrachloretheylene, and hexachlorethane to be ineffective in removing fringed tapeworms from the livers of feedlot lambs.

Ryff, Honess, and Stoddard [11] tested lead arsenate, copper sulfate, nicotine sulfate, phenothiazine, tetrachlorethylene, n-butyl chloride, sulfaquinoxaline, quarternary ammonium compound, and bis (5-chloro-2-hydroxyphenol) methane for removing fringed tapeworms from livers. Of these, only bis (5-chloro-2-hydroxyphenol) methane showed thysanosomicidal properties.

Eveleth and Goldsby [4] reported Harshfield as having stated that he found no significant difference in liver condemnation between lots of copper sulfate-nicotine sulfate medicated feedlot lambs and unmedicated controls.

According to *The Allied Veterinarian*,[1] teniatol has reduced death losses from fringed tapeworms in lambs on heavy feeding from 4.5 per cent to 0.75 of 1 per cent, thereby saving millions for the western sheep industry. Liver condemnation in lambs at packinghouses was said to be reduced from 25 per cent to 6 per cent.

Ryff, Browne, Stoddard, and Honess [10] reported that dosages of 0.25 or 0.30 Gm. of bis (5-chloro-2-hydroxyphenol) methane** per pound of body weight removed all thysanosomes from each of two groups of 3 large sheep on full feed, whereas 1 of 3 sheep that received 0.2125 Gm. retained a single tapeworm. When similar sheep were medicated after being held off feed for twenty-four hours, these authors stated that 0.05 Gm. of the medicament removed all the thysanosomes from one group of 3 sheep, whereas in two other similar groups 0.045 and 0.1 Gm. per pound of body weight removed them from 1 and 2 sheep, respectively. They considered 0.05 Gm. of medicament per pound of body weight a practical level for removing thysanosomes from sheep that had been fasted.

On the basis of these results, Ryff *et al.*[10] tested teniatol at the rate of 0.045 Gm. per pound of body weight on a large number of unfasted Wyoming feedlot lambs. In 683 medicated sheep, 6.35 per cent of the livers were condemned as compared with 24.1 per cent in 193 unmedicated lambs from a neighboring locality. In the same study, they observed an average of 16.8 per cent (11.2 to 24.1

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* An aqueous suspension of 2,2'-dihydroxy-5-5'dichlorodiphenyl methane prepared and marketed by Pitmore-Moore, Indianapolis, Ind.

** The chemical formula of the active ingredient is teniatol.

per cent) of liver condemnations in five lots of unmedicated lambs totalling 1,396 animals from five different localities.

Following medication with 0.04 Gm. of teniatol per pound of body weight of five lots totalling 3,455 sheep from undesignated areas, Ryff *et al.*[20] reported that an average of 5.3 per cent (5 to 6 per cent) of the livers were condemned at slaughter. The 1,396 unmedicated lambs with 16.8 per cent infection mentioned above were used as controls in this experiment.

MATERIALS AND METHODS

The lambs used in this study originated from two different bands in Wyoming. They were purchased by the Department of Animal Husbandry, Colorado A. & M. College, for use in feeding experiments. The animals used in 1951 came from one band and those used in 1952 came from another band. Presumably, all the individuals in each group were exposed equally to infection by fringed tapeworms during their grazing experience. Upon arrival at the college feedlots, the sheep were divided into lots, identified with numbered ear tags, and placed in separate pens for the duration of the experiment. The number per lot ranged from 27 to 50 at the time of medication.

Feed was withheld for about sixteen to eighteen hours before and for about four hours after each treatment. The animals in each lot were divided at random into two groups of approximately equal numbers. One group was medicated and the other group served as controls. Postmortem examination was made a few days later.

One fluid ounce of teniatol suspension per animal was given to six lots in 1951 and to 11 lots in 1952. In 1951, five lots received 2 oz. of the suspension.

At the time of slaughter, each liver was examined by a federal meat inspector and the writer. Only the presence or absence of tapeworms was recorded.

ANALYSIS OF DATA

Medication of lambs with teniatol gave variable results in the removal of fringed tapeworms from the livers.

Dosages of 1 oz. of teniatol suspension per lamb were slightly under the amount recommended by the manufacturer, whereas 2 oz. slightly exceeded it for some lambs but closely approached it for others.

Liver condemnation in the different lots

TABLE 1—Condemnation of Livers of Feedlot Lambs with Fringed Tapeworms, (Thysanosoma Actinoides) Following Medication with One Ounce and Two Ounces of Teniatol

			Medicated Lambs			Unmedicated Lambs			
Lot No. (1951)	Medicament per lamb (oz.)	Mean wt. lambs entire lot (lb.)	Livers with tapeworms (No.)	Total lambs (No.)	Livers with tapeworms (%)	Livers with tapeworms (No.)	Total lambs (No.)	Livers with tapeworms (%)	Livers condemned (%)
1	1	100	0	14	0.0	1	17	5.9	0.0
2	1	105	0	15	0.0	0	11	0.0	0.0
12	1	103	0	24	0.0	1	23	4.3	0.0
17	1	107	1	22	4.5	2	25	8.0	56.2
19	1	119	2	21	9.5	6	28	21.5	34.9
20	1	112	2	26	8.3	6	24	25.0	33.2
Total		107	5	122	4.1	16	128	12.5	32.8
(1952)									
1	1	103	1	13	7.7	4	14	28.5	27.0
2	1	103	1	14	7.1	3	12	25.0	28.4
4	1	109	7	20	35.0	16	23	69.5	50.3
6	1	107	1	23	4.3	2	23	8.7	49.4
8	1	98	3	23	13.0	8	23	34.8	37.3
10	1	85	7	23	30.4	8	25	32.0	95.0
12	1	98	2	23	8.6	7	24	29.0	29.6
17	1	116	0	23	0.0	8	27	29.6	0.0
18	1	102	4	23	17.3	3	25	12.0	100.0
19	1	117	0	22	0.0	4	21	19.0	0.0
20	1	110	2	22	9.0	9	24	37.5	24.0
Total		104	28	229	12.2	71	241	29.3	41.6
Total 1951-1952		104	33	351	9.4	87	369	23.8	39.5
(1951)									
4	2	108	2	22	9.0	6	23	26.0	34.6
6	2	94	1	21	4.7	1	18	5.5	85.4
8	2	87	0	19	0.0	1	26	3.8	0.0
10	2	95	1	22	4.5	2	24	8.3	54.2
18	2	103	2	25	8.0	0	24	0.0	100.0
Total		97	6	109	5.5	10	115	8.8	62.5
Grand Total 1951-1952			39	460	8.5	99	484	20.5	41.4

of sheep treated with 1 oz. of teniatol ranged from 0 to 100 per cent. The condemnation of livers for all the sheep that received 1 oz. of medicament in 1951 was 32.8 per cent, and in 1952 it was 41.6 per cent. The percentage of condemnation for the total number of sheep in both groups for the two years was 39.5 (table 1).

In the five lots of sheep that received 2 oz. of the drug, condemnations also ranged from 0 to 100 per cent but for the entire 109 animals it was 62.5 per cent (table 1).

Condemnation of livers for the 460 sheep that received 1 oz. or 2 oz. each of teniatol during the two years was 41.4 per cent (table 1).

The results obtained from medication of lambs with 1 and 2 oz. of teniatol were subjected to the *chi*-square test. With 1 oz. of medicament, the discrepancies between the observed and the expected numbers in the 1951 group are significant at the 2 per cent level, and in the 1952 group they are highly significant. The discrepancies between the observed and the expected number in the group that received 2 oz. of medicament are insignificant, even at the 5 per cent level. The pooled results of the *chi*-squares tests for all three groups with three degrees of freedom are highly significant at the 5 per cent level, thus indicating that the difference in liver condemnations between the medicated and the unmedicated lambs was not due to chance in dividing the sheep but to the action of the medicament.

Examination of table 1 shows six lots (1951: 1, 2, 12, 8; 1952: 17, 19) of medicated lambs in which no tapeworms were found at the time of slaughter. On the basis of the number of infected animals in the unmedicated controls in 1951, the expected number of infected lambs in each of the medicated groups would be 1, except in lot 2 where it would be 0. In the cases where the expected number of infected lambs in the medicated groups was 1, it is possible that by chance no infected sheep occurred in them. In lot 17 (1952), an efficacy of 100 per cent was attained in the experimental group where the incidence of infection in the control group was fairly high. In the remaining 16 lots, condemnations ranged from 24 to 100 per cent.

In the two groups of lambs that received 1 oz. of teniatol in 1951 and 1952 and which might be considered to have been slightly underdosed, the percentage of condemnation was 32.8 and 41.6, respectively, which is markedly less than the 62.5 per cent in the lambs that were given 2 oz.

These observations suggest that the action of the drug is highly variable and unpredictable. Where the highest efficacy was expected, actually the lowest was attained.

DISCUSSION

While teniatol possesses thysanosomicidal properties, the results obtained from the two extensive studies indicate a degree of efficacy so low as to make its use economically infeasible (table 2). Moreover, it showed a great variability of efficacy in our studies, ranging from 0 to 100 per cent (table 1).

Ryff *et al.*[10] medicated 683 lambs in which 6.35 per cent, or 43, livers were condemned as compared with 24.1 per cent in 193 control animals. On this basis, the expected premedication number of infected livers is 165. Thus, 26 per cent of the prob-

TABLE 2—Cost of Medication of Feedlot Lambs with Teniatol for Removal of Fringed Tapeworms (Thysanosoma Actinoides) Versus Value of the Livers Recovered for Human Consumption

No. sheep observed	Expected No. infected livers	Livers: Condemned in treated sheep (No.)	Livers: Salvaged due to treatment (No.)	Value of livers salvaged 40c* each ($)	Volume medicament required 1 oz. per sheep (Gal.)	Cost of medicament for flock* ($)	Excess cost medicament over salvage value of livers ($)	Source of information
5534	980	315	665	266.00	44	415.80	149.80	Ryff *et al.*[9]
944	194	81	113	45.20	8	75.60	30.40	This study
Total 6478	1174	396	778	311.20	52	491.40	180.20	

* $10.50 per gal. less 10% in 8-gal. lots.

ably infected lambs still harbored tapeworms after medication. If the average natural infection of 16.8 per cent reported by them [10] for 1,396 unmedicated sheep were used, the number of infected livers in the 683 animals would have been 114. The 43 condemned then would have been 37 per cent of the livers originally infected.

In a second group of 3,455 lambs that were medicated, a premedication infection of 16.8 per cent was assumed,[10] which would be 580 infected livers. Postmortem examination showed 5.3 per cent, or 183, of the livers infected with tapeworms. On this basis, the number of livers condemned was 31.5 per cent of the expected number had they not been treated.

Inasmuch as each lot of these sheep originated from different areas, it is possible that the percentage of infection with fringed tapeworms varied greatly from that assumed by Ryff *et al.*[10] Newsom and Cross [7] and Newsom and Thorpe [8] pointed out that while all the sheep in one flock may be infected, tapeworms may be absent in other flocks. They concluded that the place of origin of the lambs may be the deciding factor.

If, as indicated in our studies, 20.5 per cent of the unmedicated lambs had infected livers, then 194 infected livers should be expected in the entire lot of 944 sheep. With a liver condemnation 41.4 per cent as great in the medicated animals as in the nonmedicated (table 1), treatment of the entire group should have resulted in 81 livers condemned and 113 salvaged.

As pointed out by various workers,[2, 5-8, 10] there is no evidence that fringed tapeworms are detrimental to the health of sheep or that they cause death in these animals. The only demonstrable economic consideration to date resulting from their presence appears to be the condemnation of infected livers. In the absence of sound evidence that fringed tapeworms affect sheep other than render the livers unfit for human consumption, medication must be justified on the value of the livers that are salvaged as a result of it.

Using the information on the efficacy of teniatol obtained by Ryff *et al.*[10] from 5,534 sheep and by us from 944 animals, it becomes apparent from table 2 that medication on the basis of liver salvage was economically unsound under these conditions. Additional costs not included in the table are freight, labor, and possible losses that may accrue from fasting and handling the sheep.[12] In our case, 1 animal died in the process of medication. Even if every infected liver in the 6,478 animals, based on the above calculations, had been salvaged, their total value of 40 cents each [10] would have been $21.80 less than the cost of the drug (table 2).

However, livers that are condemned for human consumption, because of fringed tapeworms, still retain a salvage value. Thus, the loss from condemnation would not be 40 cents per liver, but the difference between that and the salvage value. This would increase the disparity between the cost of treatment and the over-all saving to the industry.

Moreover, it is doubtful that benefits can be claimed on the grounds that species of parasites other than fringed tapeworms are removed because that can be accomplished more effectively by less expensive drugs of established parasiticidal value.[3]

That teniatol could reduce death rates from 4.5 per cent to 0.75 of 1 per cent,[1] due to removal of tapeworms in sheep on heavy feed where enterotoxemia occurs, is not borne out by the results of these two studies where 26.4 to 41.4 per cent of the livers of the infected sheep retained tapeworms after medication.

Of the drugs that have been tested for the removal of fringed tapeworms from the liver of sheep, only teniatol containing 2,2'-dihydroxy-5-5' dichlorodiphenyl methane has demonstrable thysanosomicidal properties. In the light of our present knowledge regarding the pathogenicity of these parasites in sheep and the low thysanosomicidal efficacy of the drug, wholesale use of it for the removal of tapeworms is difficult to justify on an economic basis.

SUMMARY

1) Teniatol gave variable results when administered to feedlot lambs. Condemnations for 351 sheep treated with 1 oz. of teniatol was 39.5 per cent as great as for the controls; for 109 animals treated with 2 oz., condemnations were 62.5 per cent as great as for controls; and for both lots the average was 41.5 per cent.

2) Medication for salvage of livers alone was not economically feasible in these experiments.

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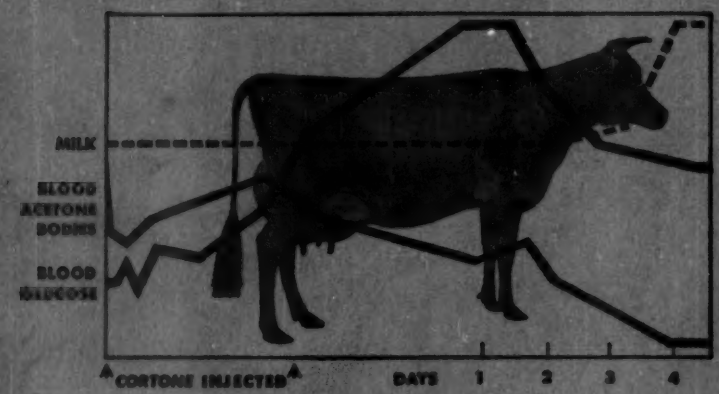

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